DOCTOR OF PHILOSOPHY

Clinical study of the use of Photodynamic Detection (PDD) in assessing suspicious oral lesions

Al-Juboori, Jamal Noori Ahmed

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University of Dundee

CLINICAL STUDY OF THE USE OF PHOTODYNAMIC DETECTION (PDD) IN ASSESSING SUSPICIOUS ORAL LESIONS

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BDS, MS

Thesis submitted for the degree of Doctor of Philosophy to the

Faculty of Medicine, Dentistry and Nursing,

University of Dundee

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University of Dundee

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DEDICATION

To my parents,

To my supporting wife,

To my two sons and daughter for their sacrifice

I would like to dedicate this work

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ACKNOWLEDGMENTS

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DECLARATION

I declare that the work presented in this thesis is all my own work, has not previously been accepted for a higher degree and I have consulted all references cited. The work has been carried out in the Oral Surgery and Medicine Unit, University of Dundee, Dental hospital and School, under the supervision of Professor Graham R. Ogden.

Signed……..

Date……...

Jamal Noori Ahmed Al-Juboori, BDS, MS

Statement 1

This thesis is being submitted in fulfilment for the requirements for the degree of Doctor of philosophy.

Signed……Candidate

Date…….

Statement 2

This is the results of my own independent work and investigation except where otherwise stated. Other sources are acknowledged by citation reference. A bibliography is appended.

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I hereby certify that Jamal Noori Ahmed Al-Juboori has fulfilled the condition of Ordinance Number 39 and the Regulations of the University of Dundee and is qualified to submit this thesis for the Degree of Doctor of Philosophy.

Signature…….....

Date……

Professor Graham R. Ogden

Head of the Unit of Oral Surgery and Medicine

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Professor David Bearn

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Abstract

Photodynamic Detection (PDD) is a diagnostic technique involving administration of a photosensitizer to the targeted cells that can be stimulated by short wavelength light which then leads to emission of light at a different wavelength (lower energy). The light emitted by the cells can be detected and analysed (by a spectroscope). All cells have the innate ability (due to endogenous fluorophores) to fluoresce, termed autofluorescence. Any cellular, metabolic or structural changes can alter the fluorescence intensity peaks. In this study 5-aminolevulinic acid (5-ALA) photosensitizer prodrug was used, which is metabolised in highly active cells to protoporphyrin IX (PpIX). Excitation of a cell at 405nm wavelength (light) leads to emission of autofluorescence at 500nm and PpIX at 635nm.

The purpose of this investigation was to evaluate the use of compact fluorescence spectroscopy together with the photosensitizer prodrug 5-ALA, in assessing clinically suspicious oral lesions. To that end the followings were assessed:

- The fluorescence intensity ratio (FIR) or Red/Green ratio at 635/500nm measurements of normal anatomical sites at ten oral anatomical sites to map and create baseline readings for normal oral mucosal site fluorescence.
- The effect of participants' characteristics on the normal oral mucosal site FIR measurements.
- The use the fluorescence intensity ratio (FIR) measurements to determine any differences between the lesion and the normal oral readings and whether the FIR from clinically suspicious oral lesions is associated with the histopathology grade. In addition to the sensitivity and specificity of the technique in assessing clinically suspicious (premalignant) oral lesions for potential malignant change.

Prior to the trial commencing, approval were obtained from the Research Ethics Committee (REC), local NHS Research and Development (R&D), and Medicine Healthcare product Regulatory Agency (MHRA) and the University of Dundee Research Innovation Services (RIS).

A total of thirty five participants with clinically suspicious oral lesions were recruited in Dundee (Dundee Dental Hospital) and Glasgow (Southern General Hospital). A Photodynamic Detection method using compact fluorescence spectroscopy and 5-ALA mouth rinse was applied. FIR measurements from ten normal anatomical sites were obtained in every patient to study the variation at different normal oral sites and the effect of the

participant's characteristics on these readings. In addition, two FIR measurements were obtained from each lesion and a further one taken from normal looking mucosa well beyond the lesion boundary (i.e. more than 5mm away) prior to biopsy. The readings were compared to study the reliability, reproducibility and efficacy of the photodynamic method in detecting mucosal abnormality.

A total of 292 spectral readings obtained from normal mucosa were used to study the FIR measurements at normal oral anatomical sites. The results showed that the oral regions could be grouped into two broad categories with similar readings, the palatal and tongue readings in one group and buccal, ventral tongue, floor of the mouth, gingiva and lip mucosa on the other (essentially keratinized and non keratinized groups).

The same set of readings were further analysed to study the effect of individual characteristics (age, gender, presence of oral prosthesis, metabolic diseases, smoking and alcohol consumptions) on the FIR measurements. There was no significant difference between FIR measurements within each of the groups studied, although at times sample sizes were very small.

A total of 134 spectral readings obtained from 47 lesions that were biopsied from 35 patients recruited to the trial were used for the next part of the study. There were 91 spectra obtained from the lesions and 43 spectra obtained from the normal sites (more than 5mm away from the borders of the lesion) for comparison. There was a significant difference between the lesion and normal site readings. The FIR readings for the dysplastic lesions were significantly different when compared with the normal and benign hyperkeratoses. However there was no significant difference between dysplastic and inflammatory lesions (lichen planus, lichenoid lesions and candidal leukoplakia) on the one hand and inflammatory lesions and hyperkeratotic lesion on the other. Further analysis showed the sensitivity in detecting all the clinically suspicious oral lesions from the normal sites was 59.5% and specificity was 73.8%. The sensitivity in detecting dysplasia from normal sites was 100% and specificity 100%.

Photodynamic Detection was able to detect a difference between the oral lesions from normal mucosa (but so is the naked eye!). However there was variation in the sensitivity and specificity in detecting a range of different pathological conditions. The technique was highly sensitive in detecting dysplasia from normal mucosa but unfortunately the technique is not able to discriminate reliably between dysplasia and inflammatory lesions whose clinical appearance can be very similar. In conclusion, the photodynamic detection method used in this study would not appear to offer a reliable screening tool for the early detection of oral dysplasia/cancer. The need to consider adjunctive tests that discriminate inflammation from dysplasia is required.

LIST OF ABBREVIATIONS

ALA	Aminolevulinic Acid	**GN**	Gingiva
ATP	Adenosine Triphosphate	**HPLC**	High Performance Liquid Chromatography
BG	Back Ground	**ICF**	Informed Consent Form
CAS	Central Allocation System	**ID**	Identity
CHI	Community Health Index	**IHCGCP**	International Harmonization Conference And Good Clinical Practice
CHM	Commission On Human Medicines	**IP**	Incisive Papilla
CI	Chief Investigator	**IRAS**	Integrated Research Application System
CIF	Site Investigation File	**ISF**	Investigational Site Files
CRC	Clinical Research Centre	**KeV**	Kilovolt
CRF	Case Report Form	**LC**	Left Cheek
CSM	Committee On Safety Of Medicines	**LL**	Lichenoid Lesion
CT	Clinical Trial	**Lp**	Lichen Planus
CTIMP	Clinical Trial Investigational Medicinal Product	**MCA**	Medicines Control Agency
DMBA	Dimethylbenz[α]anthracene	**MDA**	Medical Devices Agency
DT	Dorsal Tongue	**MDD**	Medical Devices Directorate
FDA	US Food And Drug Administration	**MeV,meV**	Referring To 1,000,000, 0.001 Electron Volt Respectively
FIR	Fluorescence Intensity Ratio	**MHRA**	Medicines And Healthcare Products Regulatory Agency
FOM	Floor Of Mouth	**MHz**	Megahertz
FN	False Negative	**OBS**	Optical Biopsy System
FP	False Positive	**OCT**	Optical Coherence Tomography
FS	Fluorescence Spectroscopy	**MP**	Mid Palate
GaN	Gallium Nitride (semiconductor)	**NADP+**	Oxidized Nicotinamide Adenine Dinucleotid (P)
GHz	Gigahertz	**NADPH**	Nicotinamide Adenine Dinucleotid (P) Hydrogenase (Reduced)

NHS	National Health Service	**S1**	Singlet State
NPV	Negative Predictive Value	**OE**	Oral Erythroplakia
OLL	Oral Lichenoid Lesions	**SD**	Standard Deviation
OLP	Oral Lichen Planus	**SOP**	Standard Operating Procedure
OLR	Oral Lichenoid Reaction	**SSA**	Site Specific Application
OSCC	Oral Sequamous Cell Carcinoma	**SSI**	Site Specific Information
PBG	Porphobilinogen	**SSIF**	Site Specific Information Form
PCA	Principal Component Analysis	**SWL**	Short Wavelength Light
PDD	Photodynamic Detection	**TB**	Toluidine Blue
PDT	Photodynamic Therapy	**TCTU**	Tayside Clinical Trial Unit
PI	Principal Investigator	**THz**	Terahertz
PIS	Patient Information Sheet	**TMF**	Trial Master File
PpIX	Protoporphyrin IX	**TN**	True Negative
PPV	Positive Predictive Value	**TP**	True Positive
PVL	Proliferative Verrucous Leukoplakia	**TT**	Tip Of The Tongue
R&D	Research And Development	**UV**	Ultraviolet
RC	Right Cheek	**VT**	Ventral Tongue
REC	Research Ethics Committee	**WHO**	World Health Organization
S0	Ground State		

Note; Abbreviations relevant to CTIMP are listed in chapter 2 (Acronyms).

Chapter 1

Introduction,

Review of Literature

& Aim of Study

1.1 INTRODUCTION

Suspicious oral lesions are lesions that clinically often look red, white or red and white in color and histologically may have a tendency to undergo malignant change. One of the greatest challenges the oral clinician faces is deciding whether the suspicious oral lesion is a potential cancer. Dysplastic and neoplastic lesions of the oral mucosa as well as hyperkeratotic and inflammatory lesions share many of the same clinical signs and symptoms.

Oral cancer is a worldwide problem with an estimated 405,000 new cases each year. In the European Union countries in particular, it has been estimated that 66,650 new oral cancer cases are diagnosed each year (IARC, 2004). In Scotland, the incidence rates of oral cancer are significantly higher than in other parts of the UK due to the higher rates of tobacco and alcohol consumption (Statistical Information Team, 2004; ISD 2010). The incidence of the disease is increasing in the developed and poor countries as well. WHO and many other health organizations and institutions around the world are therefore, concerned about detection and control of the disease. Cancer is also of great concern to the health science strategists, economist and politicians. It is well recognized that early detection and treatment of oral cancer improve the standard of life and the survival rates of the patients.

Surgical biopsy with histopathological diagnosis is still considered the “gold standard” in assessing pathological changes in tissue; however one of the most challenging aspects in early detection of oral cancer is in determining the optimal i.e.

the most dysplastic or cancerous location for biopsy in high risk patients. Patients at this stage usually require continuous monitoring which can be difficult in some instances (medically compromised patients), since this might involve multiple surgical biopsies. In addition it is time consuming, uncomfortable, and costly. Therefore**,** screening a large population at risk of developing the disease is not a feasible procedure. Several new alternative methods have been described to increase the visibility of mucosal lesions, such as photographs, application of iodine dye or toluidine blue.

In the last decade there has been increased interest in "optical biopsy" systems using tissue spectroscopy to establish pathological diagnosis. Fluorescence diagnostics has attracted increased interest both at a research level, and in the clinics. Several point-monitoring and imaging systems have been developed, and some have also turned into commercial products. Some of these systems rely on tissue autofluorescence, while others are combined with the administration of fluorescent tumor markers (photosensitizers). The intent of these systems is to provide a non invasive method of diagnosis, reduced health care cost, less time consuming, and in many cases, eliminating the need for surgical biopsy. In addition to that, the immediacy of diagnostic information can speed up the treatment and reduce the emotional trauma to the patient waiting for the diagnosis and formulating a treatment plan. The technique can also be used to guide the delivery of photodynamic therapy specifically to the detected abnormal area. The principal of this technique depends on the use of the spectrum of light and the wave lengths delivered to, and emitted from the tissue. The use of autofluorescence spectroscopy in vivo was first described in the mid 80’s to differentiate between

normal and malignant tissues. Since then several investigators have reported their findings with this technique and published in scientific journals. The concept is based upon the property that when cells interact with light they become excited and re-emit light at different wavelengths i.e. with varying colors (fluorescence) and this can be detected by sensitive spectrometers. All tissues fluoresce, due to the presence of inter and intra cellular fluorescent substances (fluorophores). These fluorescent spectra reflect architectural and biochemical changes occurring within the tissue. The resultant spectra not only detect the fluorescence but also are sensitive to cellular components that absorb light e.g. hemoglobin. The fluorescence can either occur as auto-fluorescence induced by light or from the application of photosensitizers e.g. Photofrin, 5- aminolevulinic acid and Foscan.

Photodynamic Detection (PDD) is a relatively recent diagnostic technique (medical technology) which involves administration of a light activating chemical (photosensitizer) to the targeted cells that is stimulated, usually by short wavelength light, then the emitted light is collected (at a different wavelength) and analysed by the spectroscope.

The principal of 5-ALA induced PpIX is that in excess it results in accumulation of intracellular porphyrins (especially of PpIX) which increases tissue fluorescence. Subsequent irradiation of the lesion with visible light, matching the main absorption peak of PpIX (405nm), causes cellular excitation then leads to red fluorescence emission from PpIX, peaking at 635nm. Spectroscopy can detect different fluorophores which provide characteristic spectra intensities. The difference in the overall fluorescence i.e. the ratio of the peaks of autofluorescence (green at 500nm)

and PpIX fluorescence (red at 635nm) i.e. between endogenous natural fluorphores and PpIX in normal and lesions under investigation (potential premalignant/malignant tissue) theoretically makes fluorescence spectroscopy more applicable to the discrimination between malignant and non malignant tissue. These fluorescence intensity ratio measurements are governed by many conditions which this thesis attempts to address.

1.2 REVIEW OF LITERATURE

In physics, the term *light* sometimes refers to electromagnetic radiation of any wavelength, whether the light is visible or not.

1.2.1 The Electromagnetic Spectrum

It is defined as the range of all possible frequencies of electromagnetic radiation. The "electromagnetic spectrum" of an object is the characteristic distribution of electromagnetic radiation emitted or absorbed by that particular object. The electromagnetic spectrum involve frequencies that extend from that of below modern radio frequencies down through to gamma radiation at the short-wavelength end, covering wavelengths from thousands of kilometers down to a fraction of the size of an atom.

The radio waves have a much longer wavelength than light waves with wavelengths ranging from hundreds of meters to about one millimeter.

Microwaves are electromagnetic waves with wavelengths ranging from as long as one meter down to as short as one millimeter, or equivalently, with frequencies between 300MHz (0.3 GHz) and 300 GHz. These have such a short wavelength that they are very easily absorbed by water. This is why they are used in microwave ovens.

The infrared part of the electromagnetic spectrum covers the range from roughly 300 GHz (1 mm) to 400 THz (750 nm). These radio/light waves have a very short wavelength; however their wavelength is longer than visible light.

The Visible Spectrum is the portion of the electromagnetic spectrum that is visible to (can be detected by) the human eye. Electromagnetic radiation in this range of wavelengths is called visible light or simply light. A typical human eye will respond to wavelengths from about 380 to 750 nm. Red, Orange, Yellow, Green, Blue, Indigo, and Violet, are the colours of the visible spectrum.

Ultra Violet is radiation whose wavelength is shorter than the violet end of the visible spectrum, and longer than that of an x-ray. These waves have very high energy and very short wave lengths; shorter than visible light.

X-Rays is the radiations have so much energy and such a short wavelength that they can go through the objects. The hard X-rays have shorter wavelengths than soft X-rays.

Gamma Rays are electromagnetic radiation of high energy and typically have frequencies above 10^{19} Hz. They have energies above 100 keV and wavelength less than 10 picometer. Gamma radioactive decay photons commonly have energies of a few hundred KeV, and are almost always less than 10 MeV in energy (Halliday, 2008; Young and Freedman 2008).

1.2.1.1 The Spectrum of Light

The visible light spectrum is the section of the electromagnetic radiation spectrum that is visible to the human eye. It ranges in wavelength from approximately 400 nm (4×10^{-7} m) to 700 nm (7×10^{-7} m). It is also known as the optical spectrum of light. The wavelength (which is related to frequency and energy) of the light determines the perceived colour. The ranges of these different colours are listed in the table below. The boundries are approximate as they blend into each other. The edges of

the visible light spectrum blend into the ultraviolet and infrared levels of radiation (Halliday, 2008).

1.2.1.2 The Visible Light Spectrum

Ordinary white light is a superimposition of waves with wavelengths extending throughout the visible spectrum. Dispersion of light can be achieved by a prism, and the band of colours is called a spectrum (Young and Freedman 2008).

The colors and wavelengths of the visible light can be tabulated as follows;

Colour	Violet	Blue	Cyan	Green	Yellow	Orange	Red
Wavelength	380-435	435-500	500-520	520-565	565-590	590-625	625-740

Table 1.2.1. The colors and the wavelengths of the visible light spectrum.

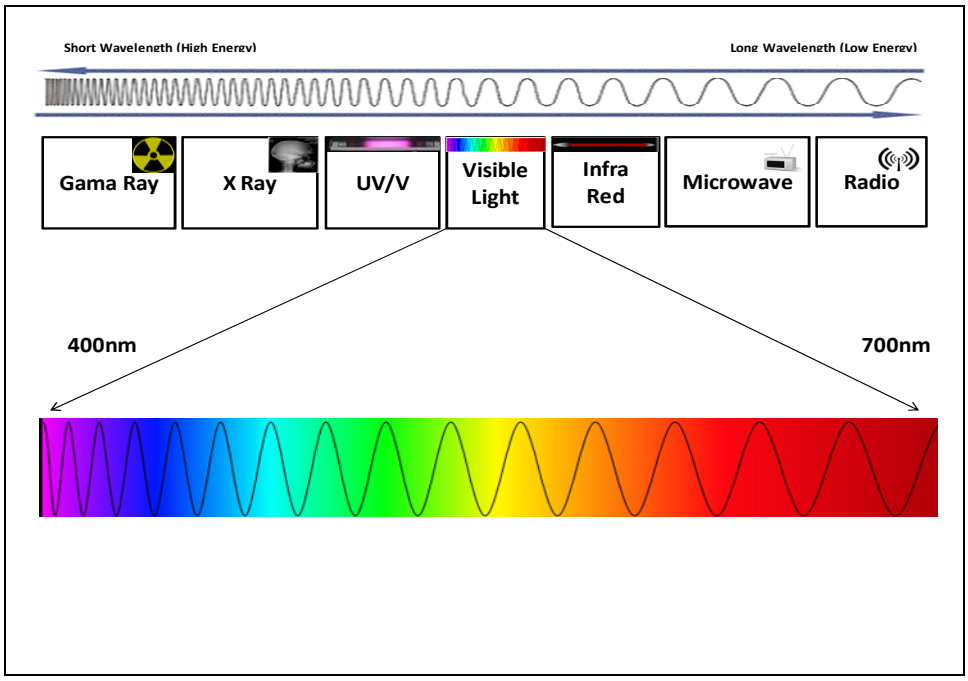


Figure 1.2.1. The electromagnetic spectrum.

1.2.1.3 Methods of Light Production

Light is produced by one of two methods;

1. Incandescence is the emission of light from "hot" matter.
2. Luminescence is the emission of light when bound electrons fall to lower energy levels.

Fluorescence is a type of luminescence which occurs when a substance absorbs radiation and begins to re-emit the radiation. This phenomenon occurs almost instantly and the fluorescent luminescence stops after the energy source is removed, that means it comes to an end as quickly as it begins. Usually, the wavelength of the re-emitted radiation is longer than the wavelength of the radiation of the substance absorbed.

The most direct way of detecting whether an atom has been exited to an upper state is to detect the fluorescence light released when the atom decays. The atom can return to the state from which it was excited via an electric dipole transition with increasing excitation energy (Svanberg, 1991).

1.2.2 The Light Interaction with Tissue

It is well known that light is essential for all forms of living activities. The light surrounding us is mainly originating from the sun. Within the biosphere, light participates in many processes. In plants, the absorption of the solar radiation by chlorophyll is a requirement for photosynthesis (Nedbal, 2007). Also in our bodies many photochemical and

photobiological reactions take place. The most obvious is the reaction with the photoreceptors in the eyes, enabling our visual perception (Collin *et al,* 2009). In the skin we can see the result of the interaction between light and tissue, as e.g. for this, is a tan following sun exposure. This is part of the protection against light that also might be harmful. The optical properties of the skin restrict the penetration depth, preventing most parts of the body from being exposed to light. Moreover, the optical properties determine the appearance of the tissue, as they are responsible for the colour and intensity of the reflected and diffusely scattered light (Gilchrest 1989; Juzenien *et al*, 2009).

The beneficial properties of light for medical treatment have been known for a long time. The ancient cultures in, for example, China, India, Egypt, and Greece have been using sun-light to treat various diseases. The introduction of light into the modern western medicine is recently introduced (Ackroyd *et al,* 2001). At the beginning of the last century, light was used in the treatment of small-pox and skin tuberculosis (lupus Vulgaris). Experiments with illumination of fluorescent drugs were also performed for the treatment of for example skin cancer, and ultraviolet (UV) radiation was found to be beneficial for patients suffering from psoriasis. A thorough review of the history of the therapeutic use of light in medicine was published (Daniell and Hill, 1991; Ackroyd *et al,* 2001). Light was also introduced for diagnostic purposes. The isolation of porphyrine and the phototoxic effect on the tumour cells led to the development of Photodetection (PDD) or photodiagnosis method (Ackroyd *et al,* 2001).

1.2.3 Photodynamic

Photodynamic is defined as the study of the activating effects of light on living organisms.

1.2.3.1 Photodynamic Technology in Healthcare

The Photodynamic method refers to the ability of certain compounds (photosensitizers**)** to destroy or inhibit the growth of living cells when exposed to light. The photosensitizer is not consumed during the process and may therefore be regarded as a catalyst, or energy transfer agent, where its primary purpose is to capture and channel the energy of light into the biological processes leading to cell death. This light-activated process can be used to destroy any type of living cell selectively, by appropriate targeting of the light, and thus photodynamic technology has numerous applications in healthcare, ranging from the treatment of medical conditions such as cancer, skin diseases and infected wounds to general sterilization of the environment and infection control. The correct choice of photosensitizer is critical for truly effective and beneficial photodynamic therapy, as it has to be readily deliverable to the relevant cells, either by systemic administration or topical application. As a result, few photosensitizers have to date achieved clinical success (Photopharmica, 2009).

1.2.3.2 Overview of the History of the Use of Photodynamics in Medicine

Sun exposure was used to treat diseases such as vitiligo, rickets, psoriasis, and skin cancer (Spikes 1985; Epstein 1990). It was not until early last century following the scientific discoveries by early pioneers such as Finsen, Raab and Von Tappeiner, utilizing phototherapy reappeared. The combination of light exposure and drug administration to the organ led to the emergence of photo-chemotherapy as a therapeutic or treatment method. The discovery of porphyrins, their tumour-localizing properties and phototoxic effects on tumour tissue led to the development of modern photodynamic detection (PDD) and photodynamic therapy (PDT) (Ackroyd *et al*, 2001).

Fluorescence diagnostics is considered a natural counterpart to photodynamic therapy, since the photosensitizers are highly fluorescent. As a result of the growing interest of fluorescence for tissue investigations, the phenomenon of fluorescence was discovered in the mid 19th century. However utilization of this property was not started until the beginning of the 20th century at which the potential of fluorescence for medical applications was being investigated. It has been reported that the photosensitizing property of haematoporphyrin was discovered by Hausmann in his report on destruction of red blood cells. In this experiment, He had noticed the symptom of photosensitisation as a side effect of the use of photosensitizer on the mice. Few years later and after injecting himself with the haematoporphyrin, Meyer-Betz noticed severe pain and swelling of the light exposed areas a few minutes after irradiation and remained

photosensitive for more than two months. He reported his finding which proved the photosensitizing properties of the agent (Meyer-Betz, 1913; Cripps, 1986; Ackroyd *et al*, 2001; Pushpan *et al,* 2002; Szeimies, 2005).

Moan and Peng (2003) mentioned in their review that there were two major advances in PDT in the period between the two world wars; *The first* finding was that Policard observed spontaneous fluorescence in experimental tumours illuminated with a Wood's lamp, and concluded that porphyrins selectively accumulate in tumours (Policard, 1924). *The second* finding was the report on photodynamic action involving haematoporphyrin on tumours published by Auler and Banzer in 1942. They investigated the selective uptake in animal tumours, and initiated some studies on humans, that were interrupted by the Second World War. This work, however, stimulated further studies on accumulation and retention of porphyrins. Figge *et al,* (1948) investigated the selective retention in vivo, and suggested the use of haematoporphyrin for cancer diagnosis. Further studies confirmed the tumour localizing properties of haematoporphyrin in a variety of tumours (Rasmussen-Taxdal *et al,* 1955; Moan and Peng (2003).

The next major step within clinical PDT was the introduction of aminolevulinic acid (ALA). Kennedy *et al,* reported on the first clinical use of topical ALA in the treatment of skin malignancies in 1990 and 1992 (Kennedy, 1992). Since then, ALA-PDT has been used in a large number of clinical specialities at various places. A review of the clinical research using this treatment modality was published and updated in the following articles

(Peng, 1997; McCaughan, 1999; Calzavara-Pinton *et al,* 2007). ALA can be used for both diagnosis (PDD) and therapy (PDT) of cancer, as will be discussed later.

1.2.3.3 Photodynamic Methods:

1.2.3.3.1 Photodynamic Therapy (PDT)

This method is also called photoradiation therapy, phototherapy, or photochemotherapy. Photodynamic therapy is now an established modality for the treatment of solid tumours and other accessible lesions (Pervaiz and Olivo, 2006). The basic principle of photodynamic method relies on a chemical reaction which involves liberation of singlet oxygen facilitated by light irradiation. This type of reaction is taken advantage of in photodynamic therapy (PDT). PDT is a modality for local treatment that has mostly been applied to malignant tumours. The technique relies on the coexistence of three components; a photosensitizer, oxygen, and light. A photosensitizer is the exogenous fluorophore or chromophore that is administered to the body, and that accumulates to a higher degree in diseased tissue. After a certain time, depending on the photosensitizing agent being used, the area is irradiated with non-ionizing radiation, usually light in the red wavelength region. A cytotoxic reaction is photo-chemically induced in the presence of oxygen and photosensitizer, leading to local cell destruction (McCaughan, 1999; Calzavara- Pinton *et al,* 2007).

1.2.3.3.2 Photodynamic Detection (PDD)

Photodynamic Detection (PDD) could be defined as a medical technology (diagnostic technique) involving administration of light activating chemical (photosensitizer) to the targeting cells that is stimulated usually by short wavelength light then collected (different wavelength with less energy) and analysed by the spectroscope.

Fluorescence diagnostics is considered as a natural counterpart to photodynamic therapy, since the photosensitizers used in the treatment are highly fluorescent, low energy or long wavelength (Red) light is required for PDT and short wavelength (Blue) light for PDD as shown in Figure 1.2.2. The mechanisms of the two methods are shown in Figure 1.2.3.

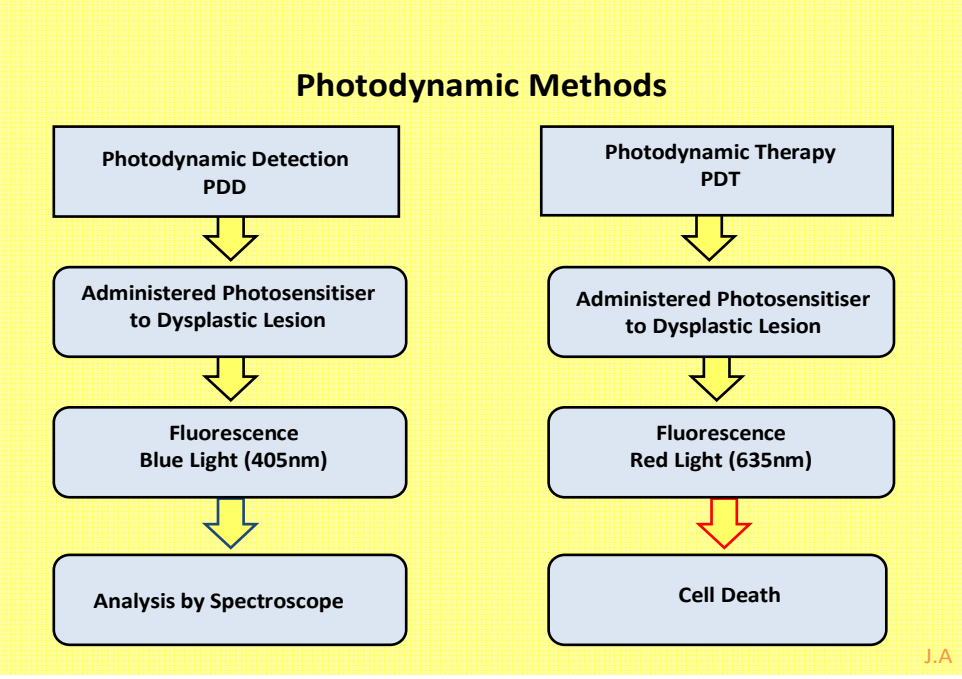


Figure 1.2.2. The steps of the two photodynamic methods (PDD & PDT) using ALA.

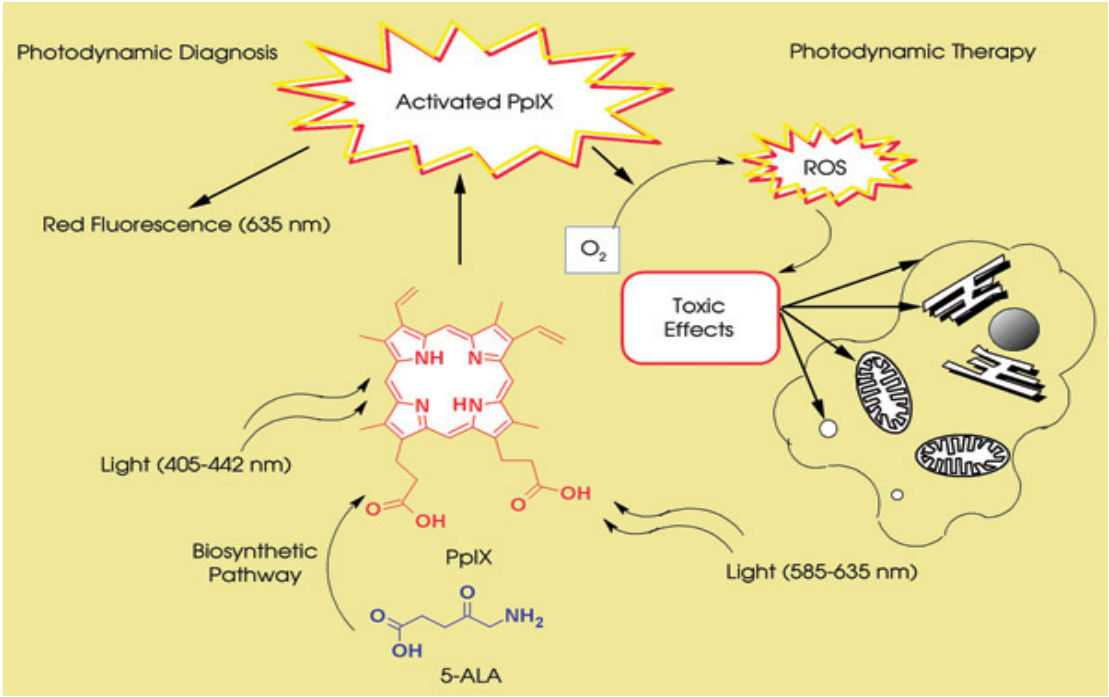


Figure 1.2.3. Light and wavelengths in PDD & PDT. Light of an appropriate wavelength (at the absorption maximum) is absorbed by protoporphyrin IX, which undergoes a transition from ground state (low energy) to the excited state. In photodynamic therapy, the activated photosensitizer interacts with oxygen to produce singlet oxygen that causes a toxic effect in tumour cells or microorganisms. In photodynamic diagnosis, the illumination of protoporphyrine IX (irradiated at short wavelength) leads to the emission of red fluorescence, while in PDT, the illumination of protoporphyrine IX with longer wavelength (low energy) lead to toxic effects (Konopka, 2007).

Optical spectroscopy techniques have in recent years been investigated in the search for novel, minimally or non-invasive methods for tissue characterization and for measurements of various parameters in the tissue. The techniques developed for tissue characterization are frequently called "optical biopsy". The methods of detecting the suspicious oral lesion using optical biopsy are shown in Figure 1.2.4.

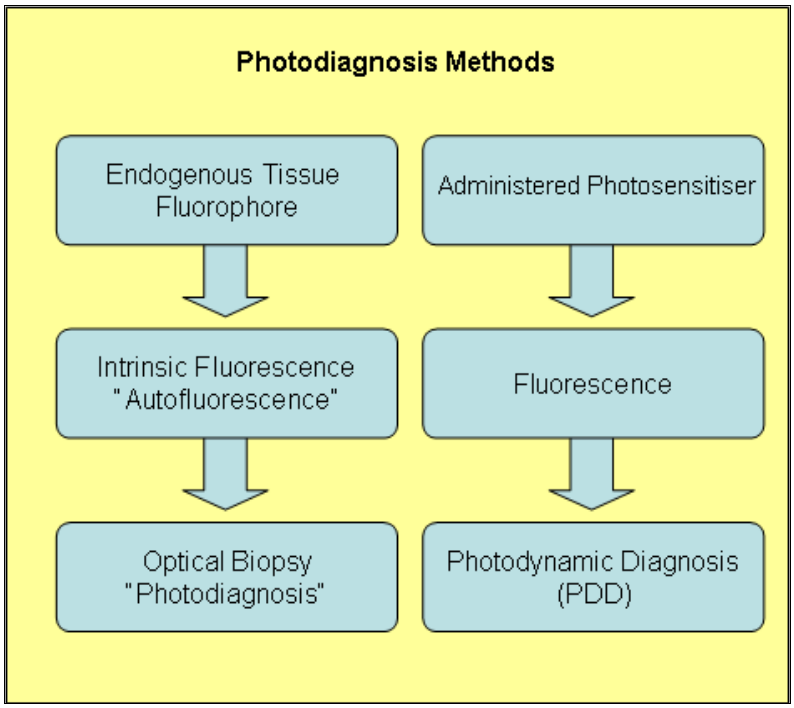
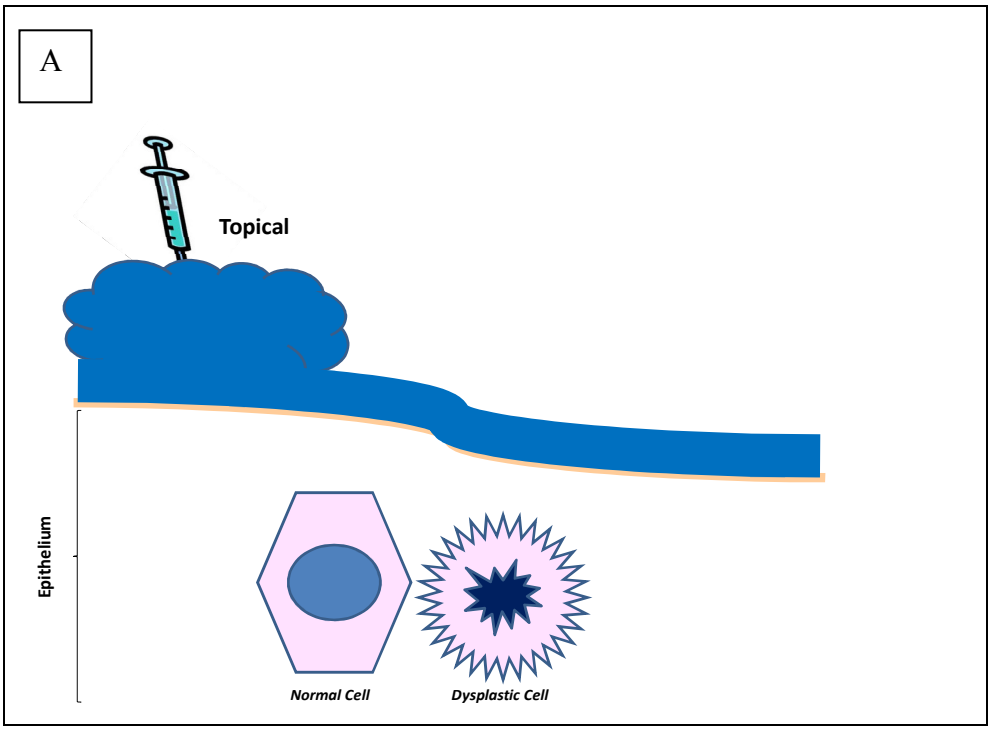


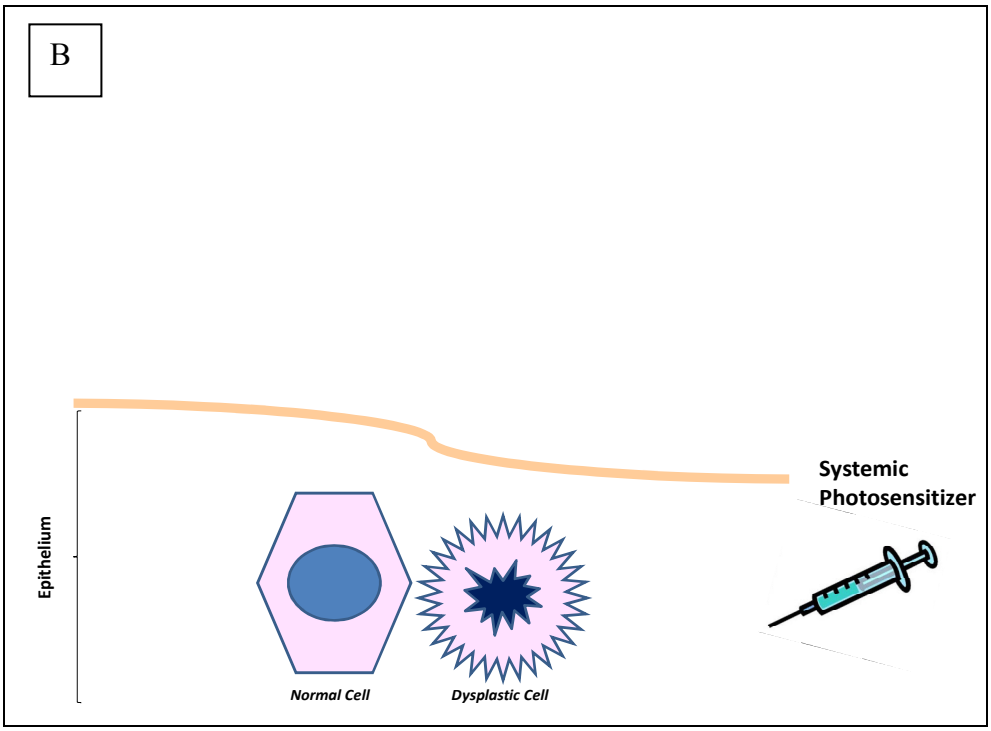
Figure 1.2.4. The methods of photodiagnosis. Optical biopsy (spectroscopy), or photodiagnosis, utilizes the fact that neoplasms cause molecular and structural changes in tissue. These changes are seen as the differences in the autofluorescence intensity between benign and premalignant/malignant tissue. In photodynamic detection, tissue fluorescence is induced by illumination of the administered photosensitizer with short wavelengths light. *(*Konopka, 2007).

To simplify the mechanism of photodynamic detection (PDD) and differentiate the procedure from the Photodynamic therapy (PDT), the author has drawn a series of diagrams explaining the mechanism of each technique in a step by step, and how do the cells react in response to each technique. The steps of the two techniques are shown in the following figures (Figures 1.2.5 A-E and 1.2.6 A-D).

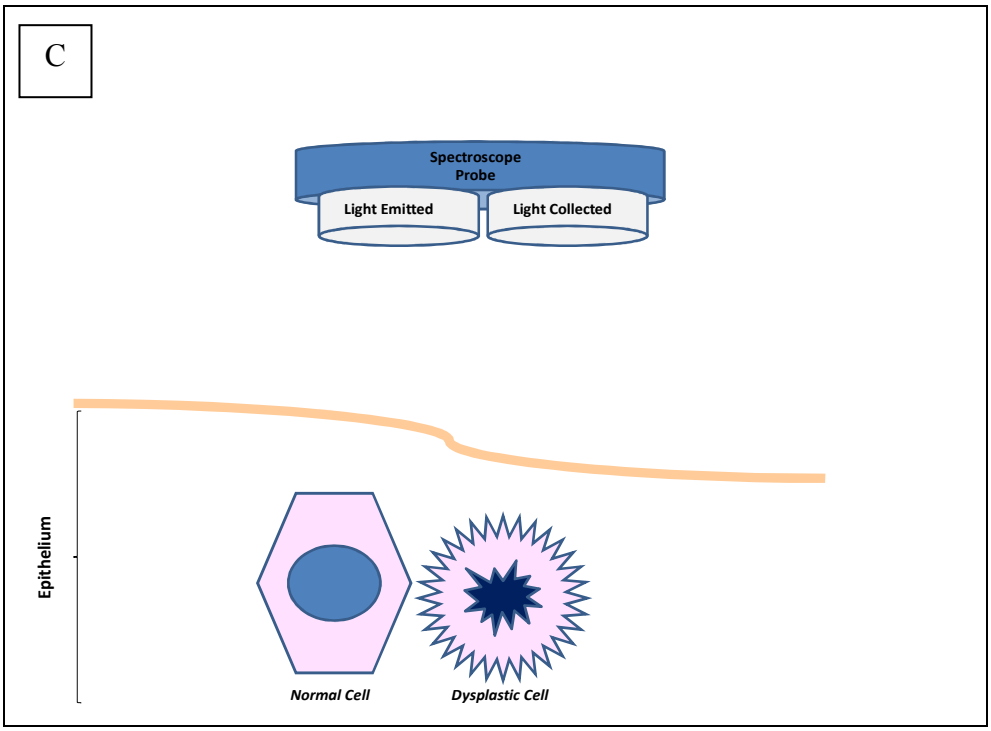
Figure 1.2.5. The mechanism of Photodynamic Detection (PDD).



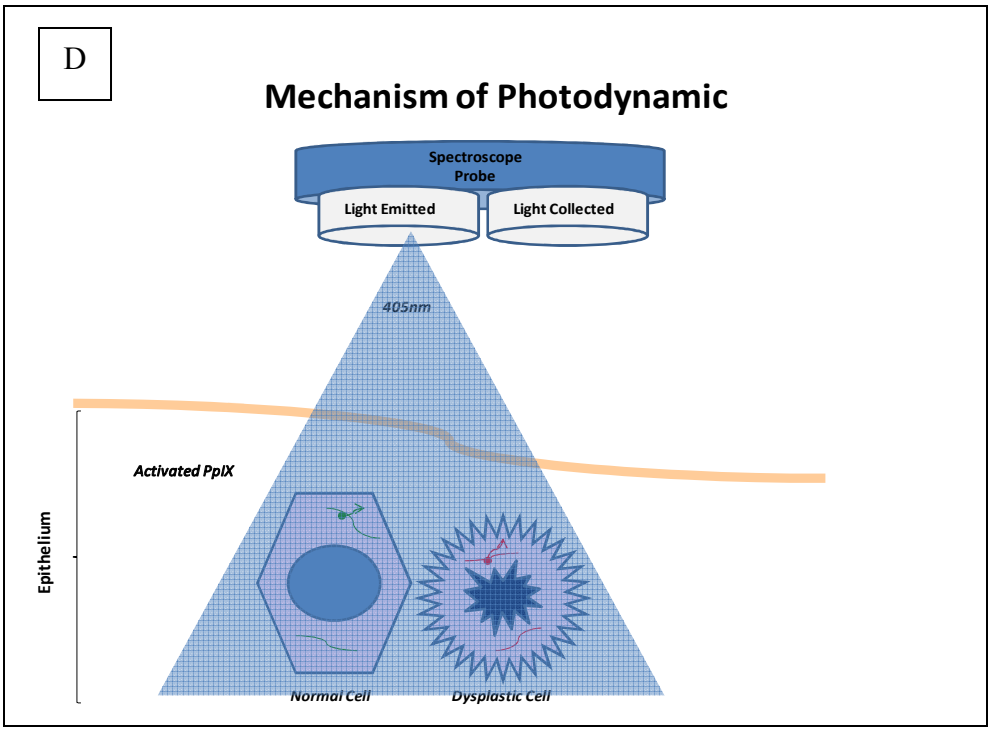
A- Topical application of the photosensitizer.



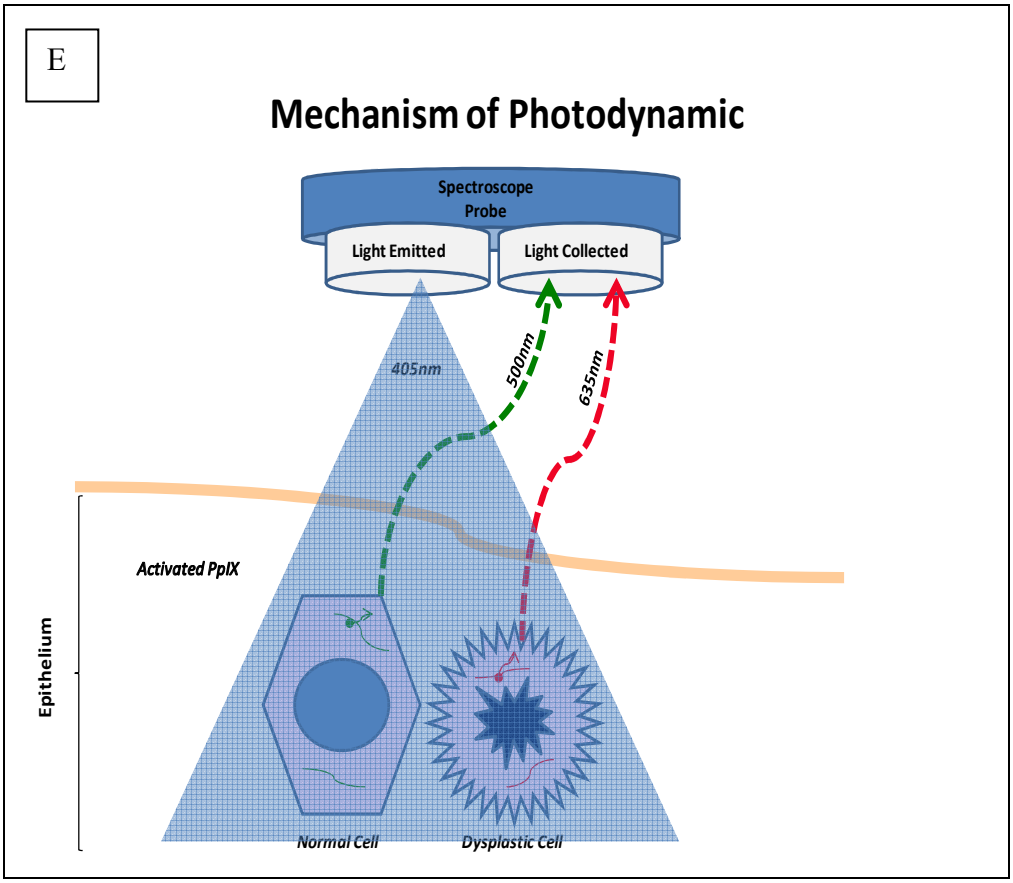
B- Application of the systemic photosensitizer.



C-Guiding the spectroscopic probe to the epithelium

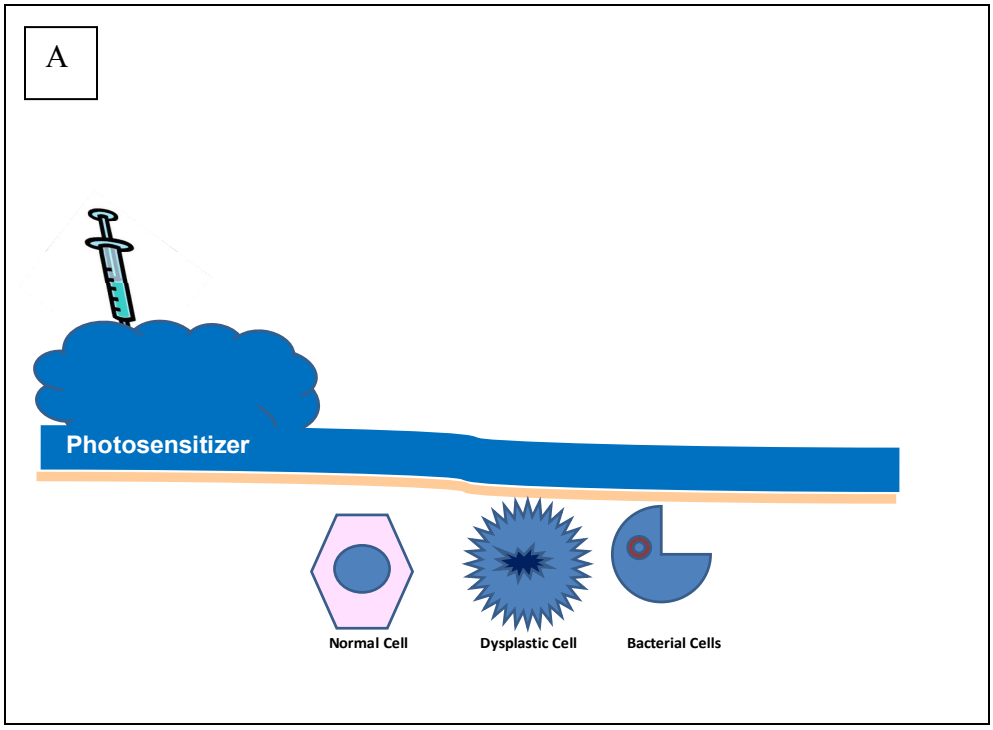


D-Excitation of fluorophores (porphyrin) and activation of PpIX in normal and dysplastic cells using short wavelength (405nm) light.

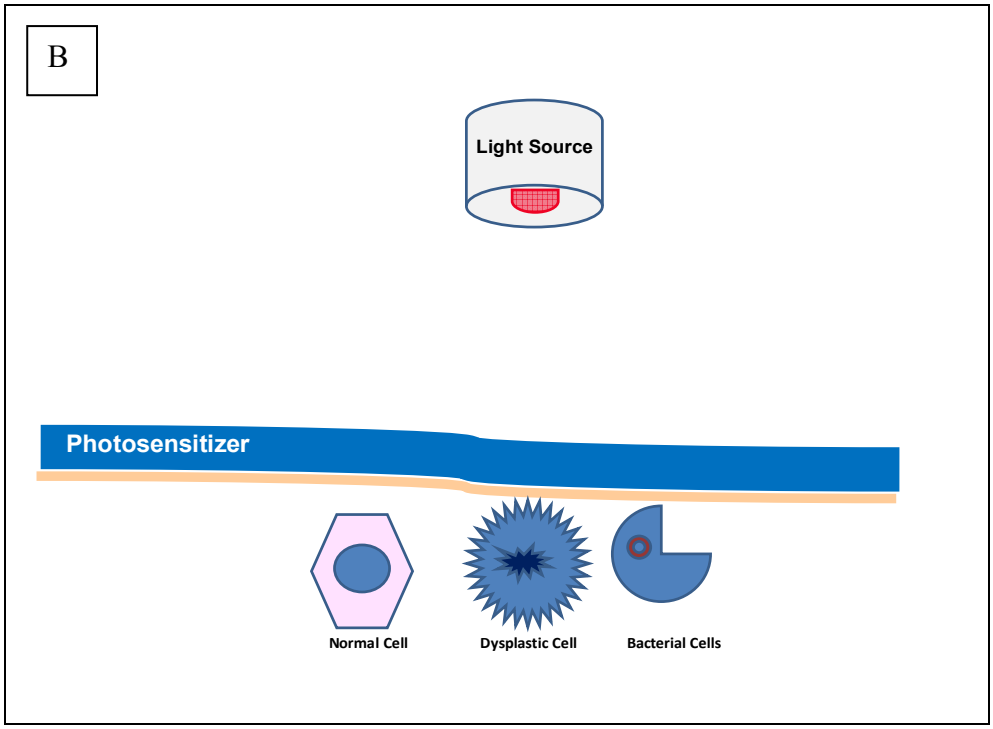


E- Emission of green (500nm) light from the normal cell and red (635nm) light from dysplastic cell collected by the probe and analysed by the computer. The ALA autofluorescence emitter at 500nm. In the dysplastic cell, ALA converts to PpIX and emits red light at 635nm.

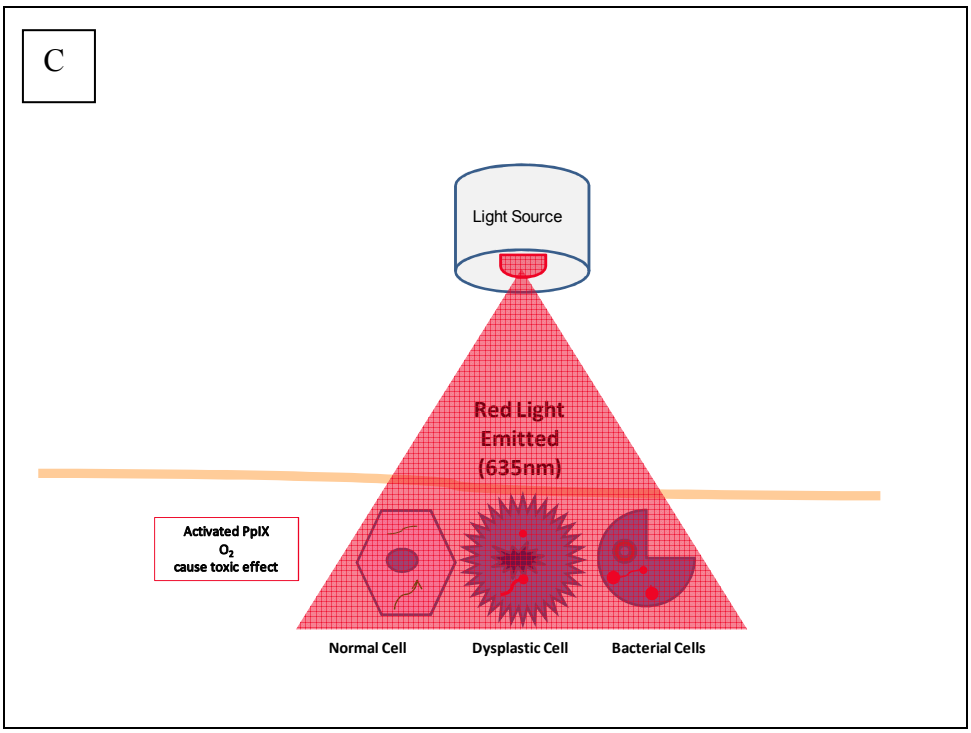
Figure 1.2.6. The mechanism of photodynamic therapy (PDT).



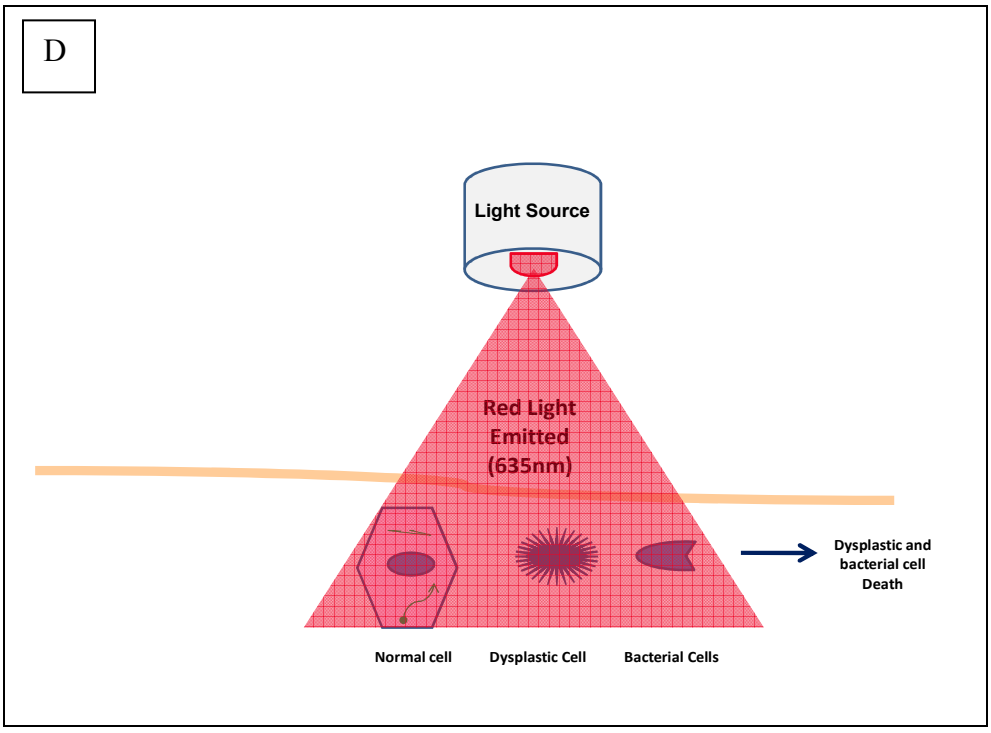
A- Application of the topical photosensitizer.



B- Guiding the light source to the lesion.



C- Exposure of the lesion to a long wavelength (635nm) light for few minutes, excitation of fluorophore (porphyrin) and activation of PpIX.



D- Death of the exposed dysplastic and bacterial cells with no effect on the normal cells.

1.2.3.4 Theory of Fluorescence Detection

Every cell of the tissues contains molecules, which have a characteristic feature to become fluorescent when excited by ultraviolet or in a violet range radiation of suitable wavelength. This fluorescence emission, arising from endogenous fluorophores, is an intrinsic property of cells and is called autofluorescence. The autofluorescence should be distinguished from fluorescent signals obtained by adding exogenous markers (photosensitizer compounds). The majority of cell autofluorescence originates from molecules inside the mitochondria and lysosomes. Together with aromatic amino acids and lipo-pigments, the most important endogenous fluorophores are pyridinic (NADPH) and flavin coenzymes. The extracellular matrix in the tissues often contributes to the auto-fluorescence emission more than the cellular component, because collagen and elastin that is present among the endogenous fluorophores have a relatively high fluorescence emission. Changes occurring inside the cell and tissue state during physiological or pathological processes result in alteration of the amount and distribution of endogenous fluorophores, chemical and physical properties of their microenvironment. Therefore, analytical techniques based on autofluorescence monitoring can be utilized in order to obtain information about morphological and physiological state of cells and tissues. In addition, auto-fluorescence analysis can be performed in real time because it does not require any treatment of fixing or staining of the specimens. Autofluorescence may actually illuminate the structures of interest in the tissues or serve as a useful diagnostic indicator (Monici, 2005).

1.2.4 Autofluorescence and Fluorophore

1.2.4.1 Mechanism of Autofluorescence

It is known that autofluorescence is the natural fluorescence of the tissue itself (auto). It is the fluorescence of tissues to which no chemical substances have been applied.

The term fluorescence in general is referred to the process by which excitation with light evokes the emission of light of a different wavelength (usually lower in energy i.e. red-shifted light). Fluorescence is the light emission resulted from a process that occurs in certain molecules called fluorophores and the process responsible for the fluorescence of fluorophores is illustrated by the electronic-state diagram (Jablonski diagram) as shown in Figure 1.2.7.

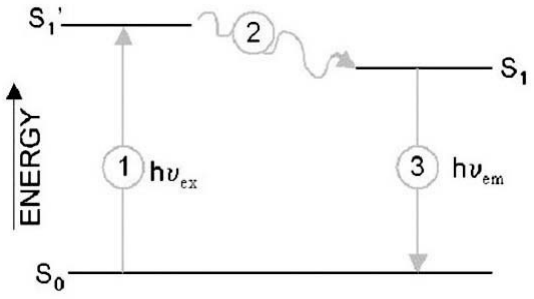


Figure 1.2.7. Jablonski diagram.

The mechanism is explained as follows;

1- A photon of energy h_{ex} (h=Planck's constant, ex =excitation wavelength) is supplied by an external source of energy such as a lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S1').

2- The excited state exists for a short time (picoseconds) during which the energy of S1' is partially dissipated, yielding a relaxed singlet excited state (S1) and from which fluorescence emission originates.

3- A photon of energy h_{em}, the emission wavelength, is emitted. The fluorophore returns to its ground state S0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower and therefore the wavelength is longer than the excitation photon h_{ex}.

The difference in energy or wavelength leads to the difference in wavelength between excitation and emission light. It allows one to easily separate the emitted fluorescence light from the excitation light. Continuous excitation of the fluorophore causes continuous emission of fluorescent light, unless the fluorophore can be destroyed by excitation or in other word called photobleaching (Elumalai *et al,* 2002).

1.2.4.2 Fluorophores

Fluorophores (chromophores) are active structures present inside the tissue molecule which causes the molecule to be fluorescence. The fluorophore which is present inside the molecule absorbs energy at certain wavelength and re-emits energy at a different wavelength. The amount of energy and wavelength emitted depend on both the chemical environment of the fluorophore and the fluorephore itself. Many types of tissue fluorophors have been identified. They have the characteristic that they can absorb light over specific wavelength bands between 250 and 500 nm and

fluorescence emission spectra within the range 300–700 nm as shown in Figure 1.2.8.

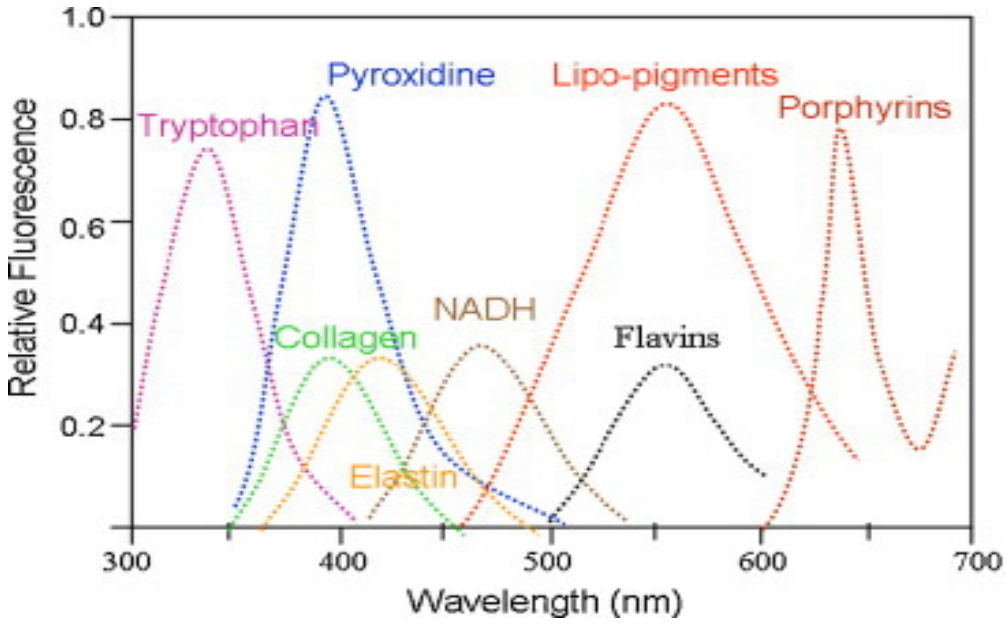


Figure 1.2.8. Normalised fluorescence emission spectra from the endogenous tissue fluorophors (Stringer *et al,* 2008).

The autofluorescence studies involve contributions from a number of fluorophors. The recorded spectrum is generally broad and lacking in specific features, therefore discrimination between diseased and healthy tissue could be achieved by identifying the differences either in emission intensity or spectral distribution, or a combination of these findings.

Collagen, elastin, the reduced nicotinamide adenine dinucleotide (NADH) coenzyme, flavins, pyroxidine, tryptophan, tyrosine, and lipopigments, as well as porphyrins are tissue endogenous fluorophores. They can be obtained from patients and in vitro tissue by morphological and functional fluorescence imaging (Richards-Kortum and Sevick-Muraca, 1996; Konig, 2000). For example, the development

from normal bronchial epithelium to invasive cancer, as illustrated in figure 1.2.9, is characterised by a progressive decrease in the overall fluorescence intensity and the intensity emission towards the end of the red spectrum. The explanation of this finding, is due to an increase in epithelial thickness (which attenuates the green light reaching the bronchial surface more than it does the red light) and an increase in blood vessel development in pre-malignant and malignant tissues (Harries *et al* 1995; Stringer *et al*, 2008).

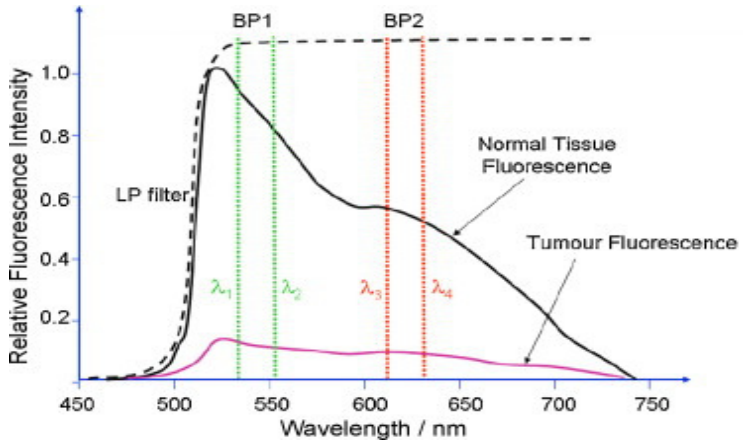


Figure 1.2.9. Fluorescence emission spectra from normal bronchial and tumour tissues (Stringer *et al*, 2008).

1.2.4.2.1 Elastin

Elastin is a protein, which is the major part of elastic fibres. These elastic fibres gives flexibility character to tissues such as the skin, lung and blood vessels (Barry *et al* 1992; Fendel and B. Schrader 1998).

1.2.4.2.2 Collagen

Collagen is a strongly fluorescent protein that forms the major component of the connective tissue. There are several different types of collagen which their absorption and emission characteristics of light are slightly different (Eyre *et al* 1984).

1.2.4.2.3 NADH/NAD+

Nicotinamide adenine dinucleotid (NADH) is the reduced coenzyme form of vitamin B3, while NAD+ is the oxidized form. NAD+ and NADH are converted into each other in numerous different metabolic activities and carry the energy in the electron transport chain, where much of the ATP bioenergy, that runs every biological process of our body, is formed. NADH/NAD+ is mostly available in the respiratory unit of the cell, the mitochondria, but could also present in the cytoplasm. In the noeplastic process where intense oxygen consumption is required for the metabolism, the oxygen level might be lower, leading to a reduced pH-value that will influence the balance between NADH/NAD+ concentrations. NADH is highly fluorescent while NAD+ is not seen when the tissue is excited above 300 nm. If the balance is pushed towards a lower concentration of NADH, this might lower the blue-green fluorescence originating from NADH (Richards-Kortum and Sevick-Muraca 1996; Drezek *et al* 2001; Pavlova *et al.* 2003; Mayevsky and Barbiro-Michaely 2009).

The blue-green autofluorescence from malignant and pre-malignant lesions has been reported lower than in healthy tissue in many *in vivo* studies (Ankerst *et al* 1984; Nordstrom *et al* 2001; De Veld *et al* 2005).

1.2.4.2.4 Other Fluorophores

Tryptophan is the precursor of a range of metabolites, which are involved in a variety of aspects of human nutrition and metabolism (Keszthelyi *et al* 2009). Diagaradjane in his report concluded that tryptophan, collagen, and NADH are the key fluorophores that undergo changes during tissue transformation process and hence they can be targeted as tumour markers (fluorescence marker) to diagnose normal from abnormal tissues using the fluorescence technique (Diagaradjane *et al* 2006). Other endogenous flurophores include flavins (Benson 1979; El-Hussein, *et al*, 2009) lipofuscin (Tsuchida, 1987), β-carotene (Andersson-Engels *et al,* 1991) and porphyrins (Alfano *et al,* 1984; Yang, *et al,* 1987) are also studied and considered as fluorescence markers.

1.2.4.2.5 Absorbers

The haemoglobin and melanin are absorbers their presence alters the recorded fluorescence spectrum emitted from the tissue. They absorb the excitation wavelength and can lower the over-all intensity of a recorded spectrum. Oxygenated (HbO2) and deoxygenated haemoglobin (Hb) have different absorption behavior. The strongest absorption peaks can be found at 414, 542 and 577 nm for (HbO2) and at 433 and 556 nm for Hb. The originated tissue fluorescence emission is absorbed

by haemoglobin and thus 'supplementary' peaks and valleys are created in the resulting fluorescence spectrum. One way to evaluate fluorescence spectra is to calculate intensity ratios at wavelengths with equal haemoglobin absorption (Andersson-Engels *et al,* 1990) as also used in the double ratio technique (Bogaards *et al* 2001) or to use a reflectance spectrum to estimate the concentration of haemoglobin and eliminate the blood interference (Muller *et al,* 2001).

1.2.4 The Photosensitizing Compounds

Many researches have been going on to investigate a variety of compounds looking for the ideal photosensitizer. There are several properties that are of interest to the investigators regarding the efficacy and usefulness of the compounds in both photodynamic methods. Some of these are:

- A high degree of selective accumulation of the photosensitizer to the neoplastic tissue.
- A high quantum yield for the generation of singlet oxygen to obtain an efficient treatment.
- near infrared region wavelength light which has good tissue penetration
- A destruction of the photosensitizer through photobleaching is required since this has no effect on the normal tissue. The low drug concentration will reduce the treatment damage threshold, in addition a very light doses is required for the therapy.

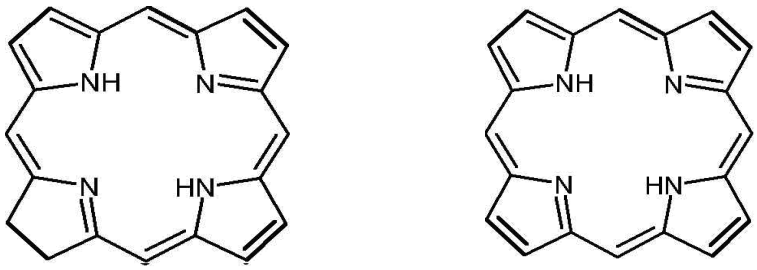
- A good tissue penetration of the compound is required to obtain active topical photosensitizer.
- Short accumulation and clearance times in the tissues.

These properties are of interest in order to minimize a general skin photosensitivity (Stapleton and Rhodes, 2003).

1.2.4.1 Porphyrins

Based on the chemical structure as shown in Figure 1.2.10, Porphyrins make up a group of molecules having photosensitizing properties. These properties and tumor selective accumulation of haematoporphyrin were investigated in the first half of the last century (Rothemund, 1936). The absorption peak of porphyrins is at about 400 nm and a number of minor peaks at longer wavelengths, up to about 630 nm. The fluorescence is characterized by a dual-peaked emission in the red wavelength region, at about 630 and 700 nm. Li et al, measured the three-dimensional fluorescence spectra of blood and stated that fluorescence peaks were attributed to endogenous porphyrins, tryptophan, NAD(P)H and the finding obtained can be used to evaluate the distorting effect of blood on the autofluorescence signals of human tissues for optical biopsy (Li *et al,* 2006). Photofrin is a partially purified form of haematoporphyrin derivative, which in turn is obtained from preparations of haematoporphyrin with acetic and sulphuric acid. It consists of a mixture of several porphyrins, monomers as well as dimers and oligomers (Ankerst, 1984).

The first health agency approval for PDT with Photofrin was obtained in Canada in 1993 for prophylactic treatment of bladder cancer (Sternberg and Dolphin, 2003). Since then, further approvals have been obtained in the United States, Japan, and some countries in Europe (Kato, 1996; Calzavara-Pinton *et al,* 2007).



Porphyrins *Chlorin*

Figure 1.2.10. The main cores of the porphyrin and chlorin structures, respectively. The difference between them is that for chlorins at least one double bond in the pyrrole rings is reduced to a single bond (Harel and Manassen, 1978; Kadish *et al,* 2003).

1.2.4.2 Chlorin

Meta-tetrahydroxyphenylchlorin (mTHPC), known under the trade name of Foscan. One well-known member of this group of molecules is chlorophyll (Erkoç and Erkoç 2002). The chemical structure of this molecule shows one or more double bonds in the pyrrole rings are reduced to a single bond, so is considered as a reduced porphyrins (Figure 1.2.10). One major difference between the two groups is that chlorins have the strongest absorption peak in the red part of the spectrum (640-700 nm) which gives the compound a green colour. Several chlorins compounds have been investigated as possible photo sensitizers for PDT. The most active of all photo sensitizers studied so far is foscan (Meta-tetrahydroxyphenylchlorin). The light doses needed for a

successful treatment is lower than for Photofrin which makes it an attractive product and has recently undergone clinical trials for head and neck cancer (D'Cruz, 2004; Lorenz, 2009).

1.2.4.3 Phthalocyanines

This compound has become a subject for much research since been found photosensitizers with some attractive qualities. The absorption in the UV and blue part of the spectrum is low, resulting in a lower cutaneous photosensitivity (Anderson et al, 1997). The absorption spectrum reveals a single peak in the red wavelength region (670-690 nm) with the singlet oxygen quantum yield was measured at 546 nm, 630 nm, and on the far-red absorption peak, the result fluorescence spectrum exhibiting a single peak at about 700 nm (Fernandez, 1997; Turker *et al,* 2009).

1.2.4.4 Texaphyrins

The texaphyrins are considered optimal metal coordinating expanded porphyrins. They involve a new series of synthetic porphyrin analogues that show promise as drugs for use in a range of medical therapies (Sessler and Miller, 2000). Texaphyrins (pentaazadentate macrocycles) have interesting photophysical properties and potential applications as photosensitizers. They exhibit a high efficiency in generating singlet oxygen, therefore could potentially be a photosensitizer and emitter for photodynamic detection and therapy applications (Lu *et al,* 2008).

Texaphyrins are a new class of porphyrin-like photosensitizers. These compounds have a high absorbance peak in the near-infrared ranges between 730-770 nm. The long-wavelength absorption peak allows the treatment of thicker lesions, due to the increased penetration of light at these wavelengths (Kostenich, 1997).

1.2.4.5 Aminolevulinic Acid (ALA)

During the last few years, many research activities were published within the field of photodynamic detection and photodynamic therapy utilizing the fluorescence properties of the tissues. Investigations have been performed using the photosensitizer prodrug b-aminolevulinic acid (ALA) in their techniques. The mechanism of ALA metabolism in the tissues is explained that when the drug is administered to living tissue, it participates in the biochemical reactions. It takes part in the haem-cycle, where it will be converted to the fluorescent and photodynamically active compound named protoporphyrin IX (PpIX) (Kennedy and Pottier 1992).

Protoporphyrins have a chemical structure of tetrapyrroles which contains 4 methyl, 2 propionic and 2 vinyl side chains. It is produced by oxidation of the methylene bridge of protoporphyrinogen. PpIX is an intermediate in heme biosynthesis, combining with ferrous iron to form protoheme IX, the heme prosthetic group of hemoglobin. Protoporphyrin IX is created by the enzyme protoporphyrinogen oxidase. The enzyme ferrochelatase converts it into heme. Protoporphyrin IX is the only naturally occurring isomer and occurs in small amounts in faeces. It is accumulated and excreted excessively in the faeces in

protoporphyria and variegate porphyria, in addition it is responsible for the brown pigment (Ooporphyrin) of birds' eggs. Protoporphyrin IX is used as a branch point in the biosynthetic pathway leading to Heme (by insertion of iron) and chlorophylls (by insertion of Mg and further side-chain transformation). Protoporphyrin IX can be used to treat liver disorders, mainly as the sodium salt. It has shown anti-HIV activity (Human Metabolome Database, 2010).

Studies on the porphyrin biochemistry have earlier been performed in relation to porphyric diseases. Berlin and his colleagues investigated the pharmacokinetics of radio-labelled ALA in humans and rats (Berlin *et al,* 1956). It is known that ALA is a naturally occurring precursor of heme. The native compound is not a photosensitizer, but in certain types of cells and tissues, it is metabolized to the photosensitizer PpIX in the body (Kennedy and Pottier, 1992). Intracellular porphyrin localization and photo-activation have been reported to occur first within the plasma membrane. After several hours of incubation, redistribution within the cell occurs to include the nuclear membrane and other organelles such as mitochondria and lysosomes (Berns *et al,* 1982; Shulok *et al,* 1990).

***Metabolism of ALA*:**

The properties of porphyrins and their metal complexes depend upon their ability to participate (mediator) in the oxidation reactions process. The chlorophylls (magnesium–porphyrin) are compounds that act as central receptors to solar energy utilisation, whilst haem (protoporphyrin IX iron chelate) acts to all biological oxidations. This compound has vital functions such as transport and storage of

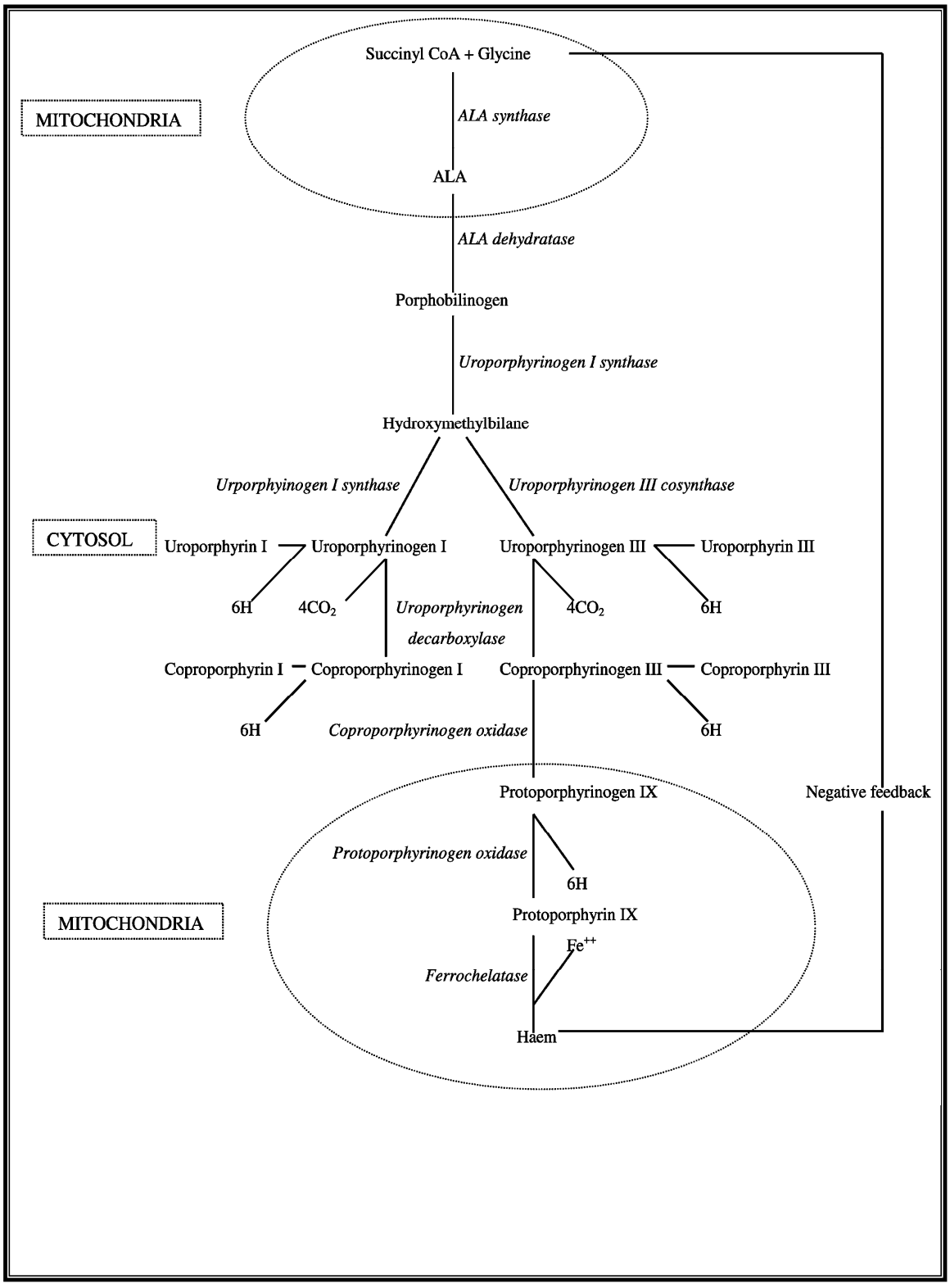
oxygen, generation of cellular energy, detoxification, regulation of protein synthesis and cell development. Haem (iron protoporphyrin of haemoglobin) is synthesised in all mammalian cells, with the exception of red blood cells, which do not possess mitochondria (Kennedy and Pottier, 1992).

The mechanism and heam biosynthesis pathway is described as follows;

"The initial substrates in this pathway are succinyl CoA, which is derived from the citric acid cycle present in mitochondria, and the amino acid, glycine. A condensation reaction between these two substrates results in the formation of ALA in mitochondria. This step, catalysed by the enzyme ALA synthase, is rate limiting, tightly regulated and controlled by regulatory feedback inhibition (Rimington 1966; Marriott 1968)**.** In the cytosol two molecules of ALA are condensed by the enzyme ALA dehydratase to form two molecules of water and one of porphobilinogen (PBG). The formation of a tetrapyrrole occurs by condensation of four molecules of PBG in a head-to-tail manner to form a linear tetrapyrrole, hydroxymethylbilane, a reaction catalysed by uroporphyrin I synthase, also known as porphobilinogen deaminase. Hydroxymethylbilane cyclises spontaneously to form uroporphyrin I, or is converted to uroporphyrinogen III by the combined action of uroporphyrinogen I synthase and uroporphyrinogen III cosynthase. Under normal conditions, the uroporphyrinogen formed is almost exclusively the III isomer, although in some circumstances the type I isomer is also formed in excess, for example in deficiencies of uroporphyrinogen III cosynthase. Uroporphyrinogen III is converted to coproporphyrinogen III by decarboxylation of all the acetate groups giving rise to methyl groups. The reaction is catalysed by uroporphyrinogen decarboxylase, which is also capable of converting uroporphyrinogen I to coproporphyrinogen I.

Coproporphyrinogen III then enters the mitochondria, where it is converted to protoporphyrinogen IX and then to protoporphyrin IX (PpIX). Several steps appear to be involved in this conversion: the mitochondrial enzyme coproporphyrinogen oxidase catalyses the decarboxylation and oxidation of two propionic side chains to form protoporphyrinogen; then oxidation of protoporphyrinogen to protoporphyrin is catalysed by another mitochondrial enzyme, protoporphyrinogen oxidase. The final step in haem synthesis involves the incorporation of ferrous iron into protoporphyrin in a reaction catalysed by haem synthase, more commonly known as ferrochelatase, another mitochondrial enzyme. This represents a second rate limiting step in the pathway to haem synthesis, regulated by a further negative feedback system". In addition, the diagram of the ALA metabolisim and the heam biosynthesis pathway as shown in Figure 1.2.11 has been published by Kelty et al, (2002).

Figure 1.2.11. Haem biosynthesis pathway *Ref* (Kelty et al, 2002).



Studies on the distribution and pharmacokinetics of porphyrins stated that the compound accumulate rapidly in the mucosal layer, however slowly in the underlying muscular tissues (Loh *et al,,* 1993; Ackroyd *et al,* 1999). Other studies have stated that Protoporphyrin IX is a potent photosensitiser and demonstrated that sufficient PpIX is synthesised by exogenous ALA administration to produce a photodynamic effect following exposure to light, both in vitro and in vivo (Malik and H. Lugaci 1987; Divaris *et al,* 1990; Loh *et al,* 1993; Leveckis 1994; Pottier *et al*, 1996; Wyld, *et al,* 1997). It was also found that Photodynamic effect correlates with the intensity of PpIX fluorescence as observed by microscopy (Divaris *et al,* 1990).

1.2.5 Excitation Light Sources

The excitation light sources used for photodynamic detection can be divided into;

- lamps
- Lasers.

There are several factors that need to be considered in choosing the light source.

- The size of the lamp should be suitable for operability of the system.
- The size of the lamp should be suitable for portability of the system.
- Low heat generation.
- Suitable for the detection technique.
- The wavelength should be appropriate for the in vivo detection application.

The wavelengths at 337 - 410 nm are of special interest in the excitation of tissue autofluorescence and porphyrin fluorescence. One of these wavelengths is 405 nm. It coincides with the band of porphyrins, and is thus of special interest for PDD (Kwasny and Mierczyk, 2003).

Wood's lamps were introduced and used in early work. They were then replaced by high-pressure mercury lamps and xenon lamps.

The xenon lamp has a broad spectrum in the near- UV region, and needs to be filtered with an optical filter or a monochromator to give an appropriate excitation wavelength.

Lasers have been introduced recently. They have several advantages over lamps for many applications because of:

- Narrow wavelength region with high intensity emission can be achieved.
- Highly paralleled beam allows efficient coupling of the light into optical fibres.
- The continuous wave ranging from ultra violet (short wavelength) to Infra red (long wavelength) which are of special interest in the excitation of tissue autofluorescence and porphyrin fluorescence respectively (Babilas *et al,* 2006; Caplan, 1967; Murtagh, 1985; Princeton instruments, 2010).

1.2.6 Fluorescence Spectroscopy for Tissue Characterization

Fluorescence spectroscopy is a non-invasive technique which has the potential to characterise different tissues. Investigations have demonstrated the importance of this technique in the detection of the soft tissue lesions. It has the ability to delineate the borders between normal and malignant tissue and to guide to the most likely diseased tissue during the clinical examination for the purpose of biopsy sampling especially in those clinical specialities, where malignant tissue cannot be visualised or diagnosed easily by the eye (Alian *et al,* 1994).

Instrumentation based on laser-induced fluorescence spectroscopy, elastic scattering spectroscopy, Raman spectroscopy, and optical coherence tomography is presently being tested and compared with punch biopsies as the "gold standard. These techniques have shown the ability to identify dysplastic or malignant regions of tissue that would not be visible to the clinician (Bohorfoush, 2000).

The information from a fluorescence spectrum can be difficult to interpret since the fluorescence emission is dominated by a few fluorophores with broad and overlapping spectra. Mathematical multivariate tools are often required to separate the fluorescence from different fluorophores and to build models for tissue classification. The use of externally applied fluorescent tumour markers can enhance the discrimination substantially. Further, different fluorophores have different absorption peaks and fluorescence instruments utilising multiple excitation wavelengths may provide additional information (Wang and Mizaikoff , 2008).

Clinical fluorescence spectroscopy of tissue was introduced as a diagnostic tool for malignant lesions in the 80's and since then; several studies have been conducted pertaining to many various clinical applications (Profio *et al,* 1984).

1.2.6.1 Optical Spectroscopy

The traditional biopsy is still the gold standard for the diagnosis of oral lesions. The rationale for developing other techniques is to improve the detection of early premalignant changes in the oral mucosa. One of these techniques under investigation is optical spectroscopy. Optical spectroscopy or optical biopsy system (OBS) allows non-invasive physical and chemical characterisation of biological tissues. The structural and chemical composition of cells and tissues strongly influences their optical features, and therefore alterations in the optical characteristics may indicate the presence of diseased tissue. Optical spectroscopy may provide possibilities in the early detection of cancerous tissues in humans. Biochemical and structural or morphological information can be gained by measuring absorption, fluorescence, elastic scattering or Raman scattering. These Optical diagnostic techniques have become an adjuvant method to conventional detection methods for the head and neck malignancy (Upile *et al*, 2007).

1.2.6.2 Optical Biopsy Techniques in Head and Neck

The "Optical Biopsy" System (OBS) is a non invasive method for biological tissue differentiation which has been proposed as an auxiliary tool for cancer detection. It has been claimed that fluorescence spectroscopy is an efficient device

for distinguishing between normal and tumour tissues and also for differentiating dissimilar clinical lesions (Kurachi et al, 2004).

As noted above, the autofluorescence depends on biochemical, and morphological aspect in addition to the tissue architecture. In many ways it is important to determine finger print fluorescence for normal and non normal tissue.

Recently there has been increased interest in "optical biopsy" systems using tissue spectroscopy to assess if those techniques can be used as an adjunct or alternative to histopathology in defining tissue involvement. In addition to aid diagnosis, treatment and follow-up, used to guide the more accurate delivery of treatment, eg photodynamic therapy (Upile *et al,* 2007).

The main techniques currently utilized in the detection of oral lesions are:

1-Microendoscopy (morphology of the surface): Microendoscopy allows in vivo examination of the epithelium to obtain the histopathological grade image without the need to remove the tissue (Gynther et al, 2000).

2-Elastic Scattering Spectroscopy (changes on cellular and subcellular level): Elastic Scattering Spectroscopy (ESS) is a technique that generates a wavelength dependant spectrum that reflects structural and morphological change within tissues.

3-Raman Spectroscopy: A Raman spectroscopy is a form of in-elastic scattering and is generated by a shift in frequency in the incident excitation light, above and below

the wave length of the incident photones due to vibrational frequencies of the bio-molecules that constitute the tissue.

4-Fluorescence spectroscopy and imaging (biochemical changes in tissue): When cells interact with light they become excited and re-emit light of varying colours (fluorescence) and this can be detected by sensitive detectors **(**Swinson et al, 2006; Upile et al,, 2007).

5- Optical Coherence Tomography (OCT) This new technology is been under investigation and shows useful results in the head and neck lesions detection.
(Jerjes et al, 2010).

1.2.6.3 Fluorescence Detection Principles and Data Analysis Methods

The data obtained from the fluorescence investigations are spectra emitted from the tissues. The type of data is dependent upon the type of equipment used in the detection.

They are of two types;

- Point-monitoring systems used to characterize the sample in a single point form recordings of the fluorescence intensity or spectrum.
- Imaging systems gives broader field of reading records.

These two systems can give complementary information on the composition of fluorophores, and on changes due to the biochemical and structural changes. Several point-monitoring and imaging systems have been developed, and some have also turned into commercial products. Some of these systems rely on the tissue autofluorescence, while others are combined with the administration of fluorescent tumour markers. The red fluorescence emission can be used to investigate the accumulation of a photosensitizer in the sample. The detected signal can then be used as a measure on the concentration of the sensitizer in the examined tissue sample.

The approach in collecting data is to record the full spectrum of light emitted from the sample. The data collected is displayed, on the screen of the instrument, as a linegraphs distribution including the fluorescence intensity at all the recorded wavelengths of the light. At these wavelengths the data will show contribution from the tissue autofluorescence and the PpIX fluorescence.

Several techniques have been proposed for the evaluation of fluorescence spectra. They can be managed by different methods;

- One statistical approach is to use stepwise multivariate linear regression (MVLR). This method relies on the shape of the normalized spectra. On a training set, the intensities at a number of wavelengths are multiplied with different weights to obtain

discrimination between normal and diseased tissue (Madhuri *et al*, 2003; de Veld *et al,* 2004).

- Principal component analysis (PCA) and neural network analysis (NNA) has also been used in the evaluation of fluorescence spectra (Nayak et al, 2006; O'Dwyer *et al*, 2008).
- Fluorescence ratios are an approach used to avoid artefacts in the data. This technique has been extensively used for tissue characterization following the administration of fluorescent tumour markers.

A wavelength around 405 nm should be used for a good excitation of porphyrins. This is not an optimal wavelength for investigations of the autofluorescence, since most of the tissue endogenous fluorophores (as shown in figure 1.2.8) have their main excitation wavelength at shorter wavelengths, below 360 nm.

5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) fluorescence has shown high sensitivity for the assessment of soft tissue lesions. Diagnosis of the oral lesions using this technique has been used as a reliable method in detecting oral premalignant and malignant lesions in vivo using different designed instruments (Zheng *et al,* 2002; Zheng *et al*, 2004; Sharwani *et al,* 2006). Some of these instruments are commercially introduced to the markets.

The system used in the thesis was developed by Professor Miles Padgett from the Department of Physics and Astronomy, University of Glasgow. Nadeau *et*

al, (2002) 1st described this (compact fluorescence spectroscopic) tool for in vivo point monitoring of aminolaevulinic acid (ALA)-induced protoporphyrin IX (PpIX) fluorescence and autofluorescence, as a non-invasive method of differentiating normal and cancerous tissue. Their results illustrate the potential of the system to be used for fluorescence monitoring in a variety of clinical applications. Subsequently, many clinical trials on body systems were conducted at Ninewells hospital University of Dundee and some of these investigations have been published in scientific journals (Nadeau *et al,* 2002; Nadeau *et al,* 2004; Ibbotson *et al,* 2006; O'Dwyer *et al*, 2008; Eljamel *et al,* 2008; Lesar *et al*, 2009).

Details of the system will be described later in the Patients and Methods section.

1.2.6.4 The Use of Optical Biopsy Systems in the Clinical Investigations

1.2.6.4.1 Studies on Animal Samples

Alfano et al, in 1984 first described the use of autofluorescence spectroscopy in vivo to differentiate between normal and malignant tissues on the rat and mouse kidney, prostate and bladder samples. Since then several authors have registered their findings with this technique.

The optical technique was used on animal models for the detection of chemically induced carcinoma. Autofluorescence spectroscopy on specimens of normal buccal pouch mucosa (normal), epithelial hyperkeratosis (hyperkeratosis), epithelial dysplasia (dysplasia), and squamous cell carcinoma (SCC) were analysed using 330

nm excitation to discriminate the lesions. The results concluded that this technique is a useful diagnostic tool for in vivo diagnosis of oral pre-cancers and cancers in DMBA-induced hamster buccal pouch carcinogenesis model (Wang *et al*, 2003). While at the lateral border of the tongue in a hamster samples using two excitation wavelength 442-532 nm the spectra results were analyzed comparing with the histopathology diagnosis, the investigator suggested using multiple wavelength excitations for a broader tissue fluorescence investigation (Kurachi *et al,* 2005).

1.2.6.4.2 Studies on Human

The technique of autofluorescence spectroscopy has been used on human and animal in the mid 80's to detect endogenous porphyrin fluorescence in tumours of the oral cavity (Harris and Werkhaven, 1987). Several other studies were conducted to utilise the application of this technique on human in detection of early oral cancers. Dhingra et al used Laser induced autofluorescence to examine areas of oral neoplasia and contra lateral areas of normal oral mucosa. They found the differences most marked at two intensities, namely 370 nm and 410 nm, and they also noted an increase in the fluorescence in the red wavelength (>600) in malignant areas. This was felt to be due to higher concentration of endogenous porphyrins (Dhingra *et al*, 1996). Gillenwater *et al*, (1998) used autofluorescence to look at neoplastic and non-neoplastic oral mucosa and found that the fluorescence intensities were less for abnormal than normal sites. They showed that the ratio of red spectrum (>600nm) to the blue spectrum (455-490nm) was greater in areas of abnormal disease.

The usefulness of fluorescence spectroscopy (FS) was examined on 130 oral lesions. The investigators suggested that FS is useful as a tool in oral tissue

pathology diagnosis (Onizawa *et al,* 1999). Betz *et al*, (1999) reported that there are spectral differences between normal and dysplastic tissues in spectrophotometric analysis in the green spectral range. The differences were found in 94% of the cases studied. De Veld *et al* investigated the influence of anatomical location on healthy mucosa autofluorescence on volunteers. They found normalisation spectra except for the dorsal site of the tongue and vermilion border of the lip (de Veld *et al,* 2003). Using fluorescence intensity in the 502–523 nm range, the individual characteristics on healthy oral mucosa autofluorescence spectral differences were studied on volunteers. It was found that there was statistically significant difference in the gender, colour, and alcohol consumption (De Veld *et al,* 2004).

The possibility of using near-infrared autofluorescence as a diagnostic technique in cancer detection was investigated. The investigators utilized various types of normal and malignant tissue samples in their investigation. Comparing the data showed differences between measurements taken from normal tissues and that of malignant nature, which could provide the basis for detection of cancer and delineation of tumor margins (Demos *et al,* 2004).

1.2.7 Clinical Manifestations of the Suspicious (Potentially Malignant) Oral Lesions

In the title of this thesis the term suspicious is referred to all lesions that have similar clinical manifestations and potentially malignant in the differential diagnosis (the white, red, red and white and/or ulceration). This includes location of these

lesions in all sites of the oral cavity which gives the impression that the lesion might be suspicious or has premalignant potential.

In a World Health Organization (WHO) Workshop, held in 2005, the terminology, definitions and classification of oral lesions with a predisposition to malignant transformation have been discussed. The term ‘‘potentially malignant” was preferred above ‘‘premalignant” or ‘‘precancerous” (Warnakulasuriya *et al,* 2007), furthermore, it has been recommended to abandon the traditional distinction between potentially malignant lesions and potentially malignant conditions and to use the term ‘‘potentially malignant disorders” instead (van der Waal I, 2009).

In this review, attention will be paid to leukoplakia, lichen planus, erythroplakia as well as other oral lesions since these are the main lesions included and listed when formulating the differential diagnosis in this study.

1.2.7.1 Oral Hyperkeratotic Lesions (Leukoplakia)

Leukoplakia is at present defined as “A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer” (Warnakulasuriya *et al,* 2007). The World Health Organization defines the term “leukoplakia” (Greek for “white, flat area”) as “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease”. The term is a clinical description of the lesion only; the use of the term is restricted to instances when no other diagnosis can be given based on clinical appearance alone. Upon further histologic investigation, microscopic diagnosis of clinical leukoplakic lesions

can range from hyperkeratosis to dysplasia, carcinoma in situ, and invasive squamous cell carcinoma (Noonan and Kabani, 2005).

Leukoplakia was found significantly more prevalent among males. Petti reported in his systematic review including 23 primary studies from all over the world that the point estimates was approximately 2.6% (Petti, 2003). Leukoplakia may affect any site of the oral and oropharyngeal cavity. Location of a leukoplakic lesion on nonkeratinized mucosa at high-risk sites for developing oral cancer, such as the floor of the mouth, the lateral and ventral tongue, and the soft palate, increases the likelihood of malignant transformation. Clinical observation indicates that one third of oral cancer cases are associated with an adjacent area of leukoplakia (Noonan and Kabani, 2005).

Leukoplakia can be subdivided in a homogeneous type (flat, thin, uniform white in colour) and a non-homogeneous type. The non-homogeneous type has been defined as a white and red lesion “erythroleukoplakia”, that may be either irregularly flat “speckled” or nodular. Verrucous leukoplakia is yet another type of non-homogeneous leukoplakia. Although verrucous leukoplakia usually has a uniform white appearance, its verrucous texture is the distinguishing feature from homogeneous (flat) leukoplakia.

Verrucous leukoplakia is clinically indistinguishable from the clinical aspect of verrucous carcinoma. Proliferative verrucous leukoplakia (PVL) is a subtype of verrucous leukoplakia (Hansen *et al,* 1985), being characterized by multifocal presentation, resistance to treatment and a high rate of malignant transformation

(Cabay et al, 2007; van der Waal and Reichart, 2008), PVL seems more prevalent among elderly women and considered unique in its predilection (nearly four to one over men), PVL is generally diagnosed in the seventh decade of life. Studies reported that 70% to nearly 100% of PVL lesions progress to squamous cell carcinoma, with the gingiva and tongue being the sites showing the highest incidence of transformation (Batsakis et al, 1999; Silverman and Gorsky, 1997). In reviewing the cases, it was noticed that months to years may elapse from the time of initial recognition of the process to its ultimate transformation to invasive carcinoma. No apparent link between human papilloma virus and use of tobacco products has been firmly established with regard to PVL (Fettig et al, 2000). Given that PVL probably represents a disease that is multifactorial in nature, it is difficult to anticipate specifically who is at high risk for developing the condition. (Silverman and Gorsky, 1997; Van der Waal, 2009). The importance of awareness of the clinician of the colour changes is of significance to the progression of the lesion to verrucous carcinoma or squamous cell carcinoma, although the effectivness of follow up has not yet been proven (van der Waal and Reichart, 2008).

1.2.7.1.1 Frictional (Irritational) Keratosis

Another oral mucosal lesion with known aetiology is frictional keratosis. It is important to distinguish this lesion from leukoplakia. Frictional keratosis is a white lesion often found on areas close to a traumatic insult. This change may be present underneath a removable partial or complete denture or on an edentulous area that has not been restored and is used for mastication. The lesion appears “clinically” as a rough hyperkeratotic area. Frictional keratosis represents a reactive epithelial

response to trauma from mastication. No treatment is generally recommended for frictional keratosis unless the character of the lesion indicates otherwise. The recurrence rate after any type of treatment varies depending on the period of follow up (van der Waal, 2010), therefore biopsy is a step required for the treatment plan.

1.2.7.1.2 Tobacco-Associated Oral Lesions

Smoking and tobacco use is by far the most important etiologic factor in a significant number of oral mucosal lesions. The proportion of both smoking and smokeless tobacco among individuals with leukoplakia is high, and a relationship is evident between the tobacco habit and the anatomical location of the leukoplakia. Cultural and demographic differences are contributing factors in pattern of tobacco usage and oral lesions. In the Eastern and South Asian study communities were likely to combine chewing with smoking and drinking, and women exhibited the highest prevalence of oral lichen planus, oral submucous fibrosis and oral leukoplakia (Lee, *et al,* 2010). In the central European countries shows high correlation between cigarette smoking and oral leukoplakia and the incidence and mortality from oropharyngeal cancer ranks among the highest in the world (Bánóczy J *et al,* 2001). Exposure of the oral mucosa to smokeless or chewing tobacco leads to a distinct clinical and histological changes. One of the most worrisome oral mucosal disorders produced by chronic habitual use of smokeless tobacco is leukoplakia. The leukoplakic changes associated with smokeless tobacco use are clearly defined in areas of the oral mucosa that come in direct contact with tobacco.

Tobacco associated lesion looks gray-white in colour with surface fissures and indistinct borders. Its clinical appearance is characterised by a brownish-red discolouration of the oral mucosa with an irregular epithelial surface that has a tendency to desquamate or peel off (Reichart, and Philipsen, 1998). Changes in the oral mucosa following chronic smokeless tobacco use are considered premalignant changes, but the risk of developing squamous cell carcinoma from smokeless tobacco use is markedly lower than that associated with other forms of tobacco use such as cigarette smoking and betel quid chewing (Bouquot and Meckstroth, 1998). Biopsy of any residual lesions is critical, because carcinomatous transformation may occur (Kaugars *et al*, 1989).

1.2.7.2 Erythroplakia

In contrast to oral leukoplakia, the term 'erythroplakia' (erythroplasia) was used to describe red lesions of the oral mucosa (Cawson *et al*, 1996). Erythroplakia (in Greek for "red, flat area") is defined in similar way to leukoplakia as "A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease" (Pindborg *et al*, 1997; Wamakulasuriya *et al*, 2007).

It has generally been accepted that oral erythroplakia is much less common than oral leukoplakia (Scully 2004). The prevalence of the lesion ranges between 0.02% and 0.83% (Peter *et al*, 2005).

Aetiology and pathogenesis of oral erythroplakia are poorly understood. Predisposing factors are widely unknown, but it has been suggested that tobacco and alcohol use are probably involved in most cases (Scully, 2004).

The erythroplakic lesions may have a smooth and velvety surface or may also be seen with other morphological characteristics. They may have an irregular, red granular surface interspersed with white or yellow foci, which may be described as granular erythroplakia. There may be numerous, small irregular foci of leukoplakia dispersed in the erythroplakic patch, and this has been called speckled leukoplakia (Pindborg, 1963). Erythroplakic areas may also be found in association with or adjacent to areas of leukoplakia. Oral erythroplakia is soft to palpation and does not become indurated or hard until an invasive carcinoma develops in it (Bouquot and Ephros, 1995). It may be impossible to distinguish between erythroplakia, erythematous candidiasis, nonspecific inflammatory processes, and vascular lesions on clinical presentation alone, for this reason, biopsy is a must to establish a definitive diagnosis. It is not uncommon to find multiple foci of erythroplakia. Erythroplakia occurs most often on the floor of the mouth, palate, retromolar region, and tongue and generally presents in an individual in the sixth to seventh decade of life (Shafer and Waldron, 1975). Oral erythroplakia is highly of representing carcinoma in situ or invasive squamous cell carcinoma at the time of diagnosis; therefore, once erythroplakia is identified, immediate action should be taken to excise the tissue (Shafer and Waldron, 1975). Van der Waal reviewed the potentially malignant disorder and recommended that erythroplakias could be treated by cold knife or laser excision. He stated that there are no data from the literature about recurrence rate after surgical excision of erythroplakias (Van der Waal I, 2010).

1.2.7.3 Inflammatory Associated Lesions

1.2.7.3.1 Lichen Planus

Lichen planus is a chronic inflammatory lesion involves the skin as well as the oral mucosa. It is considered as mucocutaneous autoimmune disease which can affect the oral mucosa, skin, scalp, nails and genital mucosa (Sumairi *et al,* 2007). It has been reported that the disease affect middle-aged patients and more commonly seen in females than males (Sugerman *et al*, 2002). Oral lichen planus could also be seen in children although rare in incidence (Laeijendecker et *al,* 2005; Patel, 2005). The prevalence of OLP is approximately 1% (van der Waal, 2009) with variation ranges between 1.9% in the Swedish population (Axell and Rundquist, 1987), 2.6% in the Indian population (Murti *et al,* 1986) and 0.5% in a selected Japanese population (Ikeda 1991), however is a relatively uncommon mucosal disorder in Malaysia and prevalent in 0.38% of the population (Zain *et al,* 1997).

Clinically, the lesion can present as white striations (Wickham's striae), white papules, white plaque, erythema, erosion or blisters The buccal mucosa, dorsum of tongue and gingiva are commonly affected. OLP usually presents as a symmetrical and bilateral lesion or multiple lesions. It can occur in six types of clinical variants namely reticular, papular, plaque like, erosive, atrophic and bullous and some variants can co-exist in the same patient. Burning sensation and sometimes pain usually accompany the erosive, atrophic or bullous type lesion (Andreasen, 1968) (Pindborg, 1997). The clinical differential diagnoses include lichenoid drug eruptions, lichenoid lesions associated with contact hypersensitivity to restorative materials, leukoplakia, lupus erythematosus and graft versus host disease (GVHD).

Direct immunofluorescence can aid in distinguishing OLP from other lesions especially vesiculo-bullous lesions such as pemphigus vulgaris, benign mucous membrane pemphigoid and linear IgA bullous dermatosis (Ismail *et al*, 2007; van der Waal, 2009; Schlosser, 2010).

Oral lichenoid reactions (OLR) are considered variants of OLP. They may be regarded as a disease by itself or as an exacerbation of an existing OLP, by the presence of medication or dental materials. Oral and cutaneous involvements have been reported. It has been associated with numerous drugs, although only some of these have been confirmed. Drugs such as beta blockers, dapsone, oral hypoglycemics, non-steroidal anti-inflammatory drugs (NSAIDs), penicillamine, phenothiazines, sulfonylureas and gold salts have been associated with lichenoid reactions (Rice and Hamburger 2002). Other than drugs, lichenoid reactions have also been associated with dental materials. Lichenoid reaction as an allergic reaction to dental materials has been widely reported. These materials include antiseptics, local anaesthetics, ultraviolet radiation, latex gloves, rubber dams, mouthwashes and other dental hygiene materials, besides, the materials used during the restorative procedures as metals, impression materials, cements, acrylics, and adhesives (van der Waal, 2009; Restrepo and Ardila, 2010). Many studies have documented contact hypersensitivity to dental materials such as composite (Lind, 1988) and amalgam (Thornhill *et al,* 2003; Sahebjamee *et al*, 2009) presenting as lichenoid reactions. Some studies also showed improvement of symptoms or recovery of oral lichenoid lesions following replacement of causative restorations (Thornhill *et al,* 2003; Sahebjamee *et al*, 2009). In most cases, OLR are indistinguishable from idiopathic OLP, clinically or histologically (van der waal, 2009).

After carrying out updated review of the different diagnostic criteria proposed for OLLCs (oral lichenoid contacts) searching the Pubmed® and Cochrane® databases, the reporter concluded that the diagnosis of these lesions is still difficult and controversial, however, these authors consider that the histopathological study is not always necessary, except when their clinical presentation is atypical in order to exclude the existence of malignancy. On this point, they consider that the histopathological study is always necessary because these lesions do not usually fulfil all the characteristic histopathological criteria of OLP and are diagnosed as compatible with OLP or OLL (Cobos-Fuentes *et al,* 2009).

Direct immunofluorescence examination is only used as an adjunct to the above method of diagnosis and to rule out specific autoimmune diseases such as pemphigus and pemphigoid. Histopathologic features of OLP and OLR are similar with some suggestions of certain discriminatory features by some authors (Ismail *et al,* 2007). Direct and indirect immunofluorescence study and cutaneous patch test may play a role in differentiating these lesions. Although the exact aetiology is unknown, OLP is recognized as a chronic disease of cell-mediated immune damage to the basal keratinocytes in the oral mucosa that are recognized as being antigenically foreign or altered (Magro *et al,* 1997).

Although the malignant transformation does not exceed 1% of the cases, in recent years the differentiation between oral lichen planus (OLP) and oral lichenoid lesions (OLL) has become important, since the latter might have a greater malignant potential (Cortés-Ramírez *et al,* 2009; van der Waal, 2009). The erosive and atrophic types most commonly undergo malignant transformation (Barnard *et al*, 1993).

Biopsy is recommended to assess dysplasia, however malignant transformation of OLP is still controversial and further prospective studies are required.

1.2.7.3.2 Oral Candidiasis

Infection with Candida albicans is most often responsible for oral candidiasis. Candida can be routinely isolated from the oral cavity of healthy persons, with variation in relation to hygiene status (Darwazeh et al, 2010). Alteration of the oral environment due to exposure to chemotherapeutic (Epstein *et al,* 2002), radiation treatment (Azizi and Rezaei, 2009) and immunodeficiency status (Soysa *et al,* 2008) may encourages overgrowth of the oral microflora.

The classification of oral candidiasis often refers to the clinical feature of the lesion and the acute or chronic nature of the process (Holmstrup and Axell, 1990). Classification of oral candidiasis could be divided into three clinical forms: pseudomembranous candidiasis (thrush), erythematous or atrophic candidiasis, and hyperplastic candidiasis (candidal leukoplakia). Biopsy and/or culture are often necessary to confirm the diagnosis and exclude dysplasia or invasive tumour from the benign lesions (Bagan and Scully, 2008). The candidal lesion add additional complexity to the clinical diagnostic process, therefore, dentists, otolaryngologists and pediatricians should be familiar with the clinical appearances of chronic mucocutaneous candidiasis to make an accurate diagnosis (Liu, Hua, 2007).

1.2.8 Early Malignant Detection Methods

At the present time early detection and diagnosis of clinically suspicious (premalignant potential) oral lesions is still based on history of the patient and thorough inspection of all oral mucosal sites. It is suggested that visual examination of the oral mucosa is effective in reducing mortality from oral cancer in individuals exposed to risk factors. It is believed that simple visual examination and clinical experience are well known to be limited by subjective interpretation and by the potential occurrence of dysplasia and early oral squamous cell carcinoma within areas of normal-looking oral mucosa. As a consequence, adjunctive techniques have been suggested to increase our ability to differentiate between benign abnormalities and dysplastic/malignant changes as well as to identify areas of dysplasia/early OSCC that are not visible to naked eye (Fedele, 2009).

The vast majority of oral neoplasms were diagnosed from lesions aroused from the mucosal surface. The incidence of occurrence of squamous cell carcinoma is considered the highest among other oral neoplasms. Diagnosis of cancerous lesions from a multitude of other red, white, or ulcerated lesions of the oral cavity is a big challenge for the clinicians. Most oral lesions are benign, but many have an appearance that is confusing with a malignant lesion, and some previously considered benign are now classified premalignant because studies have shown them statistically correlated with subsequent cancerous changes based on the clinical and histopathologic diagnoses (Bokor-Bratić *et al,* 2004). On the contrary, some malignant lesions seen in an early stage may be mistaken for a benign change. Any oral lesion that does not regress spontaneously or respond to the usual therapeutic

measures should be considered potentially malignant or malignant unless proven otherwise (histologically) (Mashberg, 1978; Silverman *et al,* 1984).

The applications of the recently investigated diagnostic techniques in the detection of the suspicious oral lesions which include toluidine blue, cytology, brush biopsy, chemiluminescence and tissue autofluorescence is reviewed.

1.2.8.1 Toluidine Blue Staining

Toluidine blue (TB), also known as tolonium chloride, is a vital dye that is believed to stain nucleic acids. Hence, it has been used over decades as a diagnostic aid for the detection of oral cancer (Seoane Lestón and Diz Dios, 2010). TB was used for the identification of mucosal abnormalities and as a useful way of demarcating the extent of a potentially malignant lesion prior to excision (Mashberg, 1983; Epstein *et al,* 2003; Gandolfo *et al,* 2006). Analysis of current evidence suggests that TB is good at detecting carcinomas, but its sensitivity in detecting dysplasias is lower (Warnakulasuriya and Johnson, 1996). In addition, controversy exists regarding the colour perception in identification of oral mucosal lesions and subjective interpretation of mucosal staining and criteria for positive results or clinical decision making (e.g. dark royal blue versus pale blue staining) (Güneri *et al,* 2010). At the present time, TB is considered as a reliable aid and has been used as a marker to differentiate lesions at high risk of progression in order to improve early diagnosis of oropharyngeal carcinomas (Allegra *et al,* 2009).

1.2.8.2 Cytology

Exfoliative cytology (cell scrapings) technique serves as an adjunct to clinical diagnosis under certain conditions. It enables screening and provides microscopic material in patients not indicated to surgical biopsy. However, cytologic smears are used infrequently and the final diagnosis as well as the treatment plan is not established on the basis of cytologic findings alone. Smears are most helpful in differentiating inflammatory conditions, especially candidiasis, from dysplastic or neoplastic surface lesions. Ogden et al reported that quantitative cytophotometric analysis may be a useful adjunct to the detection of recurrence of malignancy, prior to it becoming visible, clinically (Ogden *et al*, 1989). They also stated that cytology may be helpful in detecting field change in oral cancer, especially if this method is used in conjunction with staining (Ogden *et al,* 1991*,* 1993). Cytology may also be helpful when ulcerations following radiation are suspicious and biopsy is not indicated in real time (Perez-Sayans *et al*, 2009).

Investigators in the re-evaluation of the oral cytology reported that, although conventional cytology may be useful in oral squamous cell carcinoma and potentially malignant lesions, liquid-based cytology gives better results, enhances both the sensitivity and specificity, and also provides material for further investigations, e.g. DNA ploidy studies, microhistology, etc (Ogden and Cowpe 1989; Tucker *et al,* 1994; Ogden *et al* 1994; Navone, 2009).

1.2.8.3 Oral Brush Biopsy

The oral brush biopsy is also known as OralCDx Brush Test system. It involves collecting epithelial sample of cells from a mucosal lesion representing the superficial and deep layers of the epithelium (Drinnan, 2000). The test is of low-risk clinical technique, therefore specifically designed to investigate mucosal abnormalities that would otherwise be large or multiple or the patients refuse biopsy. The brush is a non invasive device used for epithelial cell collection and samples, fixed onto a glass slide, stained with a modified Papanicolaou test and analyzed microscopically via a computer-based imaging system (Divani *et al,* 2009). Highly suspicious lesions with cellular morphology changes for epithelial dysplasia or carcinoma is reported as positive or atypical. Results are defined as negative when no abnormalities can be found. Modified technique using liquid based cytology with specialized oral brush improves the sensitivity and specificity and is considered as a useful tool for screening of oral premalignant and malignant lesions (Delavarian *et al,* 2010). The test is considered an intermediate diagnostic step as a scalpel biopsy must follow when an abnormal result is reported (Fedele, 2009).

1.2.8.4 Chemiluminescence

Chemiluminescence using blue or white light (ViziLite™) has been evaluated and considered as a diagnostic aid in the detection of oral cancer and potentially malignant epithelial lesions. It was suggested that chemiluminescence is a more reliable diagnostic tool than tolonium chloride in the detection of oral cancer (Ram and Siar 2005) The technique is conducted by application of oral rinse with a 1%

acetic acid solution for 30–60 seconds then the lesion examined and evaluated using a chemiluminescent light (wavelength of 490 to 510 nm) (Epstein *et al,* 2008). The theory behind this technique is that the acetic acid removes the glycoprotein barrier and slightly desiccates the oral mucosa, the abnormal cells of the mucosa then absorbing and reflecting the blue/white light in a different way with respect to normal cells. Hence normal mucosa appears blue, whereas abnormal mucosal areas reflect the light (due to increase in the nuclear/cytoplasmic ratio of epithelial cells) and appear brighter, sharper and with distinct margins (Epstein *et al,* 2006; Farah and McCullough, 2007).

1.2.8.5 Tissue Fluorescence Imaging

A number of new diagnostic aids to conventional oral examination have recently been introduced to assist in the early detection of oral neoplasia. In particular, autofluorescence imaging has emerged as a promising adjunctive technique to improve early identification of oral premalignant lesions (Shin *et al,* 2010). Laser-induced fluorescence diagnostics can be used as an imaging system for detecting premalignant and malignant lesions of oral mucosa. The technique utilizes the variation in the colour and intensity of light emitted from the tissues after excitation with short wavelength light. The concept is based on the findings that changes in the cellular structure such as pleomorphism, hyperchromatism and mitosis and thickening of keratin layer of the epithelium as well as changes of the subepithelial stroma alter the distribution of tissue fluorophores which later reflects on the behaviour of tissue autoflorescence (De Veld *et al,* 2005).

Imaging system has recently been developed by LED Medical Diagnostics Inc. in partnership with the British Columbia Cancer Agency and is marketed as VELscope system. It increases the clinician's ability to detect oral changes that may represent premalignant or malignant cellular transformation, however presence of false positive findings are possible in the presence of highly inflamed lesions (Kois and Truelove, 2006). This result was supported by Shin et al who stated that recent studies of wide-field autofluorescence imaging in low-prevalence populations suggested that benign lesions such as inflammation may give rise to false-positive results, therefore automated image analysis should be investigated to maximize overall diagnostic performance for early detection of oral neoplasia. (Shin *et al,* 2010).

1.2.8.6 Tissue Fluorescence Spectroscopy

Fluorescence spectroscopy is a technology that has shown great promise during initial investigations. Tissue autofluorescence has been used in the screening and diagnosis of precancers and early cancer of the lung, uterine cervix, skin and, more recently, of the oral cavity (Gillenwater *et al,* 1998; De Veld *et al*, 2005). The autofluorescence spectroscopy system consists of optical fibre that produces various excitation wavelengths and a spectrometer that receives and records on a computer and analyzes, via dedicated software, the spectra of reflected fluorescence from the tissue. The advantage of this technique is the elimination of the subjective interpretation of tissue fluorescence changes (Inaguma and Hashimoto, 1999; Lingen *et al*, 2008).

Various photosensitising agents have been introduced. They can be used to increase the contrast in the fluorescence signal between normal tissue and malignant and premalignant lesions. The properties of these photosensitisers are that in excess it results in accumulation of intracellular substance which increases tissue fluorescence (Onizawa *et al,* 2002).

5-Aminolevulinic acid (5-ALA) is one of these agents has shown sensitivity for the assessment of systemic and oral soft tissue lesions as well. The principal of 5-ALA induced PpIX is that in excess it results in accumulation of intracellular porphyrins or especially of PpIX which increases tissue fluorescence. Subsequent irradiation of the lesion with visible light matching the main absorption peak of PpIX (405nm) leads to red fluorescence emitting from PpIX peaking at (635nm) (Leunig *et al*, 1996; Betz *et al*, 2002). The difference in fluorescence ratio between normal and premalignant/malignant lesion makes detection and analysis by fluorescence light more applicable in the discrimination between malignant and non malignant lesion (Ebihara *et al*, 2003; Müller *et al*, 2003). Dysplastic and malignant tissues, as well as having different spectral characteristics, tend to have increased red fluorescence and decreased green fluorescence. Significant increase in the red/green fluorescence ratio is said to be an accurate predictor of oral malignancy using autofluorescence (de Vield *et al,* 2004) and 5-ALA (PpIX fluorescence) (Zheng *et al*, 2002).

The principle of using fluorescence constructed optical diagnostic instrument (spectroscopy) seems to be a useful method for distinguishing lesions from healthy oral mucosa, in general. The detection would be distinct especially when malignant tumours are compared to healthy mucosa. The technique has been under

investigation in detection of the suspicious lesions and furthermore in performing guided surgical biopsies and photodynamic therapy.

Various fluorescence spectroscopy diagnostic systems has been introduced and more researches are needed to support the clinical application in order to help experienced clinicians at improving their ability to detect premalignant and malignant lesions in high-risk individuals attending the specialised clinics. Experienced surgeons use some of the described optical diagnostic aids or probes to improve the identification of a lesion's margins and extensions in the operatory setting, although it is not known the impact these techniques have on the patients' survival and risk of disease recurrence (Fedele, 2009).

Photodynamic detection (PDD) using Compact fluorescence spectroscopy and ALA has been investigated in detecting suspicious lesions in different organ systems (in Ninewells hospital). This study investigates their application in assessing clinically suspicious oral lesions.

AIM OF STUDY

The purpose of this investigation was to:

1- Detect the fluorescence intensity ratio (FIR) measurements (ratio of PpIX fluorescence at 635nm and autofluorescence at 500nm, i.e. red/green ratio or 635/500nm) at ten oral anatomical sites to map and create baseline readings for normal oral mucosal site fluorescence.

2- Detect the effect of the participant's individual characteristics (e.g. tobacco use) on the fluorescence intensity ratio measurements taken from the normal oral mucosa.

3- Use the fluorescence intensity ratio (FIR) measurements to determine;

a- any differences between the lesion and the normal oral site readings.

b- whether the FIR from clinically suspicious oral lesions is associated with the histopathology grade.

c- the sensitivity and specificity of the technique in assessing clinically suspicious (premalignant) oral lesions for potential malignant change.

Chapter 2

Optimization the Clinical Trial Investigational Medicinal Products (CTIMP)

“Review of Issues”

When undertaken a clinical trial of an investigational medicinal product (CTIMP) the trialist should be fully aware of the scientific, administrative and regulatory compliance issues relevant to the design of such a trial in the host country. Compliance with these issues is mandatory to ensure safety of the patient. In this chapter, these issues are reviewed and the steps and measures considered in the trial explained clarifying the requirements for the CTIMP.

Clinical research may be defined as the research method(s) conducted by the investigator(s) to study the association(s) between effect of therapies experienced by participants and their health condition. A clinical trial refers to a trial to evaluate new drugs, instruments and other interventions, in addition to assessing the safety and efficacy of experimental treatment methods and assessing whether a new intervention is better than the standard therapy (Sheffield Children's NHS Foundation Trust, 2009).

The Medicines and Healthcare products Regulatory Agency (MHRA) is a government organisation which was set up in 2003 to bring together the functions of the Medicines Control Agency (MCA) and the Medical Devices Agency (MDA). Now, the MHRA also looks after blood and blood products, working with UK blood services and the healthcare providers to improve blood quality and safety.

The principal aim of the Agency is to ensure the safety of the public's health. It does this by making sure that medicines and medical devices (from painkillers to pacemakers) work properly and are acceptably safe; and by responding quickly when new incidents are highlighted. No product is completely free of risk but the MHRA's decisions are taken to ensure that these risks are minimised to acceptable level.

2.1 History of the UK regulations

In order to understand why there is such an organization as MHRA, it is helpful to be reminded of the evolution of health care regulation in the United Kingdom.

The regulation of medical devices began in the mid 1990s. The Department of Health had been established to ensure high standard of quality and safety of medical equipment. The Medical Devices Directorate (MDD) in effect became the Medical Devices Agency in 1994 which then merged with its medicines counterpart in 2003 to become the MHRA. The Committee on the Safety of Devices is an independent group of experts which advises the MHRA.

In the 1950s and early 1960s, Thalidomide was prescribed to relieve morning sickness in the first few months of pregnancy. This therapy had seriously caused unpredicted birth defects and as a result of this drug regulations in the UK were created. To prevent a similar occurrence, the Committee on Safety of Drugs was established in 1963. This subsequently became the Committee on Safety of Medicines (CSM) under the terms of the Medicines Act of 1968, which provided the legal framework for the control of medicines in the UK. In 2005 this committee became the Commission on Human Medicines (CHM).

The Medicines Control Agency was established in 1989, and merged with the Medical Devices Agency (MDA) to become the MHRA in 2003. The Commission on Human Medicines provides independent expert advice to the MHRA (MHRA 2009).

2.2 The UK clinical trial regulations

The UK clinical trial regulations were initially published in 2004. The laws were relating to the implementation of Good Clinical Practice (GCP) in the conduct of clinical trials on medicinal product for human use. The regulations were amended in 2006 to implement European directives in 2005. Other miscellaneous amendments included specific requirements to report serious breaches to the approved protocol. In 2006 second amendment created an exception of the incapacitated adults to be included in the clinical trials and established new rules for the inclusion criteria. Further amendment with respect to the ethics committee was done in 2008. These amendments were reported; however no consolidated published version is available (Canary 2004, 2006, 2008).

2.3 Good Clinical Practice (GCP)

International Ethical and Scientific Quality Standard for the design, conduct, performance, monitoring, auditing, recording, analyses and reporting clinical trials that involve participation of human subjects. In addition, assurance that the clinical trial data are credible and accurate and that the trial subjects rights, integrity and confidentiality are protected.

The World Medical Association set out the Declaration of Helsinki in 1964 describing ethical principles for medical research involving human subjects. Currently, it is necessary to obtain ethical approval for any research carried out within the NHS or University in the UK. Further and separate approvals are also

required for researches involving the use of medicinal products or equipment for human use (Clinical Trials).

Historically, the Declaration was originally adopted in June 1964 in Helsinki, Finland, in part because of human experimentation that occurred during the 2nd world war. It has since undergone six revisions (the most recent at the General Assembly in October 2008) and two clarifications, growing considerably in length from 11 to 32 paragraphs. The declaration is an important document the history of research ethics as the first significant effort of the medical community to regulate research itself, and forms the basis of most subsequent documents. Revisions were in (1975-1983-1989-1996-200) Clarifications of articles 29, 30 (2002-2004)-2008.

It is a mandatory quality assurance system that drug companies and university/hospitals have to set up to ensure that their clinical trials are performed in compliance with the national and international regulations.

Most of the international companies established their set of procedures up to the highest standards (FDA or EU GCP) to ensure that the data generated by their clinical trials will be accepted universally by all authorities. Every company is free to decide upon the content and format of their SOP 's. PharMed GCP, SOP's and Audits (Podoorsem, 2009).

In order to comply with the terms of IHC GCP (International Harmonization Conference and Good Clinical Practice) and UK legislation, the University and NHS has to ensure its research staff are aware of their responsibilities and are working to appropriate standards. The University of Dundee has accredited a range of Standard

Operating Procedures (or Guidelines) for use in Clinical Trials using Investigative Medicinal Products.

The Research Governance Framework for Health and Community Care recommends that a senior individual must be designated as the chief investigator (CI) or principal investigator (PI) for any research undertaken involving participants' or their organs, tissue or data.

2.4 Going about setting up a clinical trial

It is mandatory the PI be aware of the current regulations and should have a plan to ensure compliance with ICH GCP and other issues before setting up the trial.

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As example in this trial, the thesis author arrived in the UK and started this project in November 2007. The application process for approvals is lengthy and requires the applicant to have sound background and knowledge of the submission pathways for CTIMP. For this reason the chief investigator (CI) had submitted all the required applications and obtained the approvals from the authorities to conduct the clinical trial in 2006 in order to complete the study programme within the time frame of the UK Universities which ranges between 3-4 years.

The author gained knowledge of conducting CTIMPS by attending the workshops of IHC GCP (International Harmonisation Conference for Good Clinical Practice) before starting the trial and updated his knowledge by attending the same workshop one year later to gain a good background in GCP. In addition to all the clinical trial

seminars offered by Tayside Clinical Trial Unit at Ninewells Hospital for the staff and researchers to improve his background. Thus he become aware of the regulation and to be eligible to conduct a Clinical Trials. The attendance certificates are shown in appendix 1.

The PI and colleagues prepared the study Protocol, Trial Master File in Dundee and Site Investigation File (CIF) in Glasgow including the associated documents to set up the trial.

2.4.1 Acronyms

Commonly used acronyms were listed in the following table!

AE/SAE/SAR/SUSAR	Adverse Effect/Serious Adverse Effect/Serious Adverse Reaction/Suspected Unexpected Serious Adverse Reaction
CRF	Case Report Form
CT	Clinical Trial
CTA	Clinical trial Authorisation
CTIMP/IP	Clinical trial investigational Medicinal Product/ Industrial Product
CTU	Clinical Trial Unit
EudraCT	European Union Drug Regulating Authorities Clinical Trials
GCP	Good Clinical Practice
GMP/GPP	Good Manufacturing Practice
IB	Investigators Brochure
SmPC	Summary of Product Characteristics
IMP	An Investigational Medicinal Product or drug used in a CTIMP which may be a test drug or a comparator or Placebo
IRAS	Integrated Research Application System
ISRCTN	International Standard Randomised Controlled Trial Number
MA	Marketing Authority
MHRA	Medicines and Healthcare products Regulatory Agency
MREC	Multicentre Research Ethic Committee
PI/CI	Principal Investigator/Chief Investigator
PL	The Product Licence that confirms the drug has a MA from the regulatoryAgency
QP	Qualified Person or quality control expert employed by the manufacturer to give assurances about a drug's quality and fitness for use in practice. The process is called 'QP release'
QP (IMP)	Refers to QP release for a drug manufactured, packaged and supplied specifically for a clinical trial
QP (MA)	Refers to QP release for a drug manufactured, packaged and supplied for use in clinical practice
REC	Research Ethic Committee
SOP'S	Standard Operating Procedures
TMF/SMF/IMF	Trial Master File/ Site Master File/ Investigation Master File

2.4.2 Preparing and writing a protocol

The chief investigator (CI) of the study should prepare a protocol to conduct the trial with the content and structure of protocols for clinical trials of investigational medicinal product. The protocol should be constructing to cover important methodological considerations and requirements specified under Good Clinical Practice. The title should include a brief outline of the study design, medicinal product(s), route of administration, method of detection, patient population and setting.

2.4.2.1 Purpose of the study and aims

The protocol should state the purpose of performing the study and the impact on the patient's health. They should include the primary and secondary objectives.

2.4.2.2 Methods and study design

The credibility of results obtained the scientific methodology of a study are largely dependent upon the study design.

The study design should include;

- A description of the design of the study.
- The expected duration for which each patient will participate in the study.

- Description of all procedures to be performed in sequence, identifying what is standard and nonstandard.
- Information referenced to other documents such as the Investigators Brochure or summary of product characteristics for licensed medicinal products should be explained in details in this section.

2.4.2.3 Information added to the protocol

The following information should be clearly identified;

- Details about Full name or the trade name if licensed drug in the UK and Europe.
- Information about the - UK or EU License.
- Potential risks and benefits to patients should be summarized.
- The proposed route of administration (e.g. topical %-ALA) dosage (e.g.3%), and application period (e.g.15 min).
- Description of, packaging, labelling and dosage form of products.
- Description of disposal procedures during the trial.
- Details of the source and who will supply the products.
- The shelf life and arrangements for storage and other issues.

2.4.2.4 Patient selection

The protocol should include;

- Source of patient recruited for the trial, for example in this study, they were referred patients attending the oral medicine or oral surgery clinic having suspicious oral lesions.
- Number of centres involved in the trial. This study recruited patients in two sites in Dundee (Clinical Research Centre at Ninewells Hospital) and Glasgow (Southern General Hospital).
- The expected number of eligible participants. This protocol anticipated a total of 50 patients after statistical advice from Prof. Donnan re p of study (25 in each site), however the achieved number of 35 patients (27 at Dundee and 8 at Glasgow) could not reach the target number.

2.4.2.5 Inclusion criteria

The protocol should define who is eligible for the study. This protocol stated that inclusion criteria are all patients over 18 years of age and having suspicious (potentially malignant) oral lesion.

2.4.2.6 Exclusion criteria

The protocol should consider contra-indications to be included in the trial. This protocol involved pregnant women and patients younger than 18 years.

2.4.2.7 Patients' compliance

Recording of patient compliance and details of the follow-up should be provided. An explanation and the instruction (patient information sheet) were given to each patient recruited. A record of patient compliance information and details of follow up was done by providing the patient with contact details and two weeks review.

2.4.2.8 Randomisation or blinding

Avoiding bias was not taken by randomisation method since the study was a diagnostic test accuracy trial. The spectra results were compared with the gold standard (surgical biopsy).

2.4.2.9 Withdrawal of participants

The patient's withdrawal criteria should be written in the protocol. This should include when and how to withdraw patients or participants, the follow up procedures for withdrawn participant and data should be reported in details in the CRF.

2.4.2.10 Data collection

Detailed list of all data should be provided including.

- The Source of the data (e.g. patient questionnaires, patient notes, electronic data…etc).

- Who will collect the data (form and type of data e.g. continuous or binary...etc)?
- The data should be collected in a special form (data collection forms) and should be kept as appendices.

2.4.2.11 handling the data

The protocol should state who is responsible of collecting and recording the data. Procedures for collection, storage, duration of maintenance, security and management of data should be described including Data Protection.

2.4.2.12 Final protocol details

The following information is required to be included in the protocol:

- Sponsor
- Funder
- Chief Investigator
- EudraCT number
- CTA number
- MREC number
- Register with a Protocol registration scheme
- Version number & date

2.4.3 Approvals required for the trial

Prior to the trial commencing, approvals are mandatory and should be obtained. The requirements of the approvals are related to the design or type of the study. The CTIMP requires Research Ethics Committee, Research and Development and MHRA approval.

In order to understand the procedure, it would be useful to explain the steps that were followed for this trial and the required approvals obtained, as an example.

The CI had contacted the University of Dundee (UoD) Tayside Clinical Trials Unit (TCTU) to discuss sponsorship.

1-University of Dundee had to ensure a scientific review for the study protocol, Informed Consent Form (ICF) and Patient Information Sheet (PIS), so that the TCTU (Tayside Clinical Trial Unit) committee assess the risk of the study and possible sponsorship. After assessment of the scientific quality of the protocol and the proposed research accepted, a delegation letter was sent from TCTU to the CI confirming the clinical trials authorisation and insurance. The documents should be in place before applying for REC (Research Ethic Committee) approval. Because the legal side was evolving during the study, the insurance was put in place after the trial started. The University requires that all applications are made using the Integrated Research Application System (IRAS) online application form which could be accessed through a website (https://www.myresearchproject.org.uk) The Site Specific Information form (SSI) would then be completed to assess the suitability of

the site chosen e.g. at Dundee (Ninewells Hospital) and Glasgow (Glasgow Southern General Hospital) and of the research team at these sites.

2-Submission of Part A and B of the application form and supporting documentation direct to the REC within 4 working days of booking (getting the number) from the Central Allocation System (CAS) or Local Ethic Committee. The multi sites trial within one domain required CAS or local REC application booking. Then the clock was started for 60 days and a letter confirming the validation was sent (5 days). Because this study involved both Dundee and Glasgow, the REC application went to Glasgow.

3- Research and Development (R&D) management approval is required at each site before research can begin. An application for R&D management approval may be made alongside the application for ethics approval. For multi-site studies, early discussions were encouraged with the identified hosts.

The Site Specific Information Form (SSIF) could also be used for R&D management approval.

The responsibility for site specific application (SSA) was transferred to R&D during 2007. Final R&D approval can not be confirmed until after a favourable ethical opinion has been received.

4-Medicines and Healthcare products Regulatory Agency (MHRA) application for clinical trial authorisation form was completed and all the associated

documentations submitted for approval. The guide for the applicants is shown in figure 2.1.

2.4.4 Preparations of Trial Master File (TMF)

It is the responsibility of everyone who wants to conduct a clinical trial to establish and maintaine Trial Master File (TMF) and it should comply with the principles of good clinical practice (GCP) for clinical trials of investigational medicinal products (CTIMPs).

The University policy of the Trial Master File (TMF) is that the file should contain all the essential documents (*) and should be stored in a lockable cabinet or room.

For multicentre studies, the Chief Investigator (CI) must ensure that adequate investigational site files (ISFs) are in place at individual sites. The Principal Investigator (PI), being the lead investigator at the site person while PI is considered the CI in single site studies. The PI may delegate the task of setting up the ISF to a member of the research team and write it in a form of a document and kept in the

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*[those documents which individually and collectively permit evaluation of the conduct of a trial and the quality of the data produced and they serve to demonstrate the compliance of the investigator, and sponsor with the principles of Good Clinical Practice and with all applicable regulatory requirements. Essential Documents also serve a number of other important purposes. For example: they can greatly assist in the successful management of a trial by the investigators and sponsor, and they are also the ones which are usually monitored by the sponsor and inspected by the regulatory authority(ies)].

delegation log. The PI is responsible for updating the ISF with any relevant new documentation as the study progresses and to ensure that the ISF is made available to trial monitors and representatives of the MHRA. In addition, for reviewing the ISF to ensure that all the required documents are present and maintained for at least five years after the completion of the trial for archiving.

The PI is also responsible of all new or amended documents (apart from the subject ID log, recruitment log and consent forms) and to be issue of minor amendments, sent to the CI for the Trial Master File (TMF).

2.4.5 Records for staff training

The member of staff involved with establishing and running a clinical trial must have a Training Record kept in a TMF. It is the responsibility of the CI/PI to ensure that all staffs are suitably trained to conduct CTs and have sufficient training and efficiency to conduct the trial specific procedures. As a minimum, they must show evidence of GCP.

Good Clinical Practice (GCP) workshops in the Clinical Research Centre (CRC) or similar GCP course should be attended by all the staff and documented proof of attendance must be kept in the training record. In addition, the staff should be aware of any applicable regulatory requirements pertaining to the clinical trial conduct. Training records must be reviewed regularly to identify gaps.

2.4.6 Other documents

The protocol, patient information sheet (PIS) and informed consent form (ICF) must be approved by an ethics committee, local NHS R&D departments, the MHRA (if applicable) and any other appropriate regulatory approval bodies before potential participants are approached to enter a trial.

In accordance with the principles of GCP all subjects taking part in research studies should be fully informed about the study that they have been asked to participate in and written consent will be obtained before any study specific procedures were carried out.

[In conducting this trial, the investigators were keen to explain the procedure to the patients in detail (non scientific words) that it is completely voluntary and he or she would withdraw at any time without providing a reason and without prejudicing the future oral surgery care. They were also given PIS and the information about possible of benefits to other patients in the future. The patients were given enough time to read the information about the research this is usually at least 24 hours before the procedure.

The ICF should be on headed paper, have a version number and date. Only the most recent approved version of the ICF was used for obtaining informed consent to comply with SOP in conducting the trial. As the PIS or IC are updated, the up todate version should be recorded on the sheet, as well as in the TMF. Copies of the ICF were also kept in the dental hospital notes. The staff named on the Delegation of

Responsibility Log are allowed to obtain informed consent from participants, although mistakenly at the start, a couple of cases were signed by unauthorized staff member (their names were not on the delegation Log), all the required measured were taken to ensure no further breach to the trial Standard Operation Procedure].

The ICF should be signed and dated by the participant and the person obtaining informed consent as witness at the time of conducting the procedure making sure the patient is fully aware of the trial and happy to participate. The original copies of the ICF should be kept in the patient's note. Moreover, the patient need consent to the taking of photos.

The trial investigators should check and update the confidentiality measures of the obtained documents in the TMF, i.e. not including the name, age and CHI number of the patient. The confidential information is kept in the CRF.

2.4.7 Some other considerations

There are certain considerations should be taken in account:

- Risk Assessment & Trial Monitoring
- Delegation of responsibilities when absent
- Protocol amendments & violations
- Closure & Archiving

2.5 Pharmacovigilance: What are these?

NHS Tayside acted as the Pharmacovigilance sponsor. The role is delegated to the Pharmacovigilance section of Research Development Services based at Ninewells Hospital & Medical School. Investigators are requested to notify any SAEs or SARs 'immediately'. 'Immediate' means at the earliest convenient time after the person has become aware of the event, if possible within 24 hours.

- Adverse event (AE)
- Serious Adverse Event (SAE)
- Adverse Reaction (AR)
- Serious Adverse Reaction (SAR)
- Suspected Serious Adverse Reaction (SSAR)
- Suspected Unexpected Serious Adverse Reaction (SUSAR)
- Define serious?
- Who checks for this?

Measures should be considered when SUSAR occurs;

- The initial report should be submitted by email.
- A signed copy of the Detailed Written Report should be sent by post.
- The detailed written report, which should follow as soon as possible thereafter, will contain all of the information that is required by the sponsor for onward reporting to MHRA and the Ethics Committee.

The investigator should also retain a copy of the detailed follow-up report in the CRF.

2.6 Role of trials pharmacist regarding drug

These measures should be taken in account:

- Procedures for Manufacturing & Assembly
- Packaging & Labelling & Storage
- Investigators Brochure
- Has the drug Marketing Authorisation (MA)? NB original packaging
- Otherwise Trial specific Qualified Person release
- Note Exemption 37 in hospital trial protocol amendments & violations
- Substantial amendments

e.g. add or delete PI

- Any change in Protocol

e.g. Gliolan v raw product, change to inclusion criteria

2.7 Closure and archiving

An End of Trial Study Report should be sent to the MHRA and relevant Ethics Committee within 12 months of the end of the trial. The summary of the final report may be enclosed with the end of study declaration (sent within 90 days of the end of the trial, or within 15 days if the project is terminated early), or sent to the MHRA and relevant ethics committee subsequently. Titles of these activities are shown in Appendix 1 (Series of seminars and workshops introduced by Tayside Medical Science Centre (TASC) and Tayside Clinical Trial Unit TCTU 2007-2010).

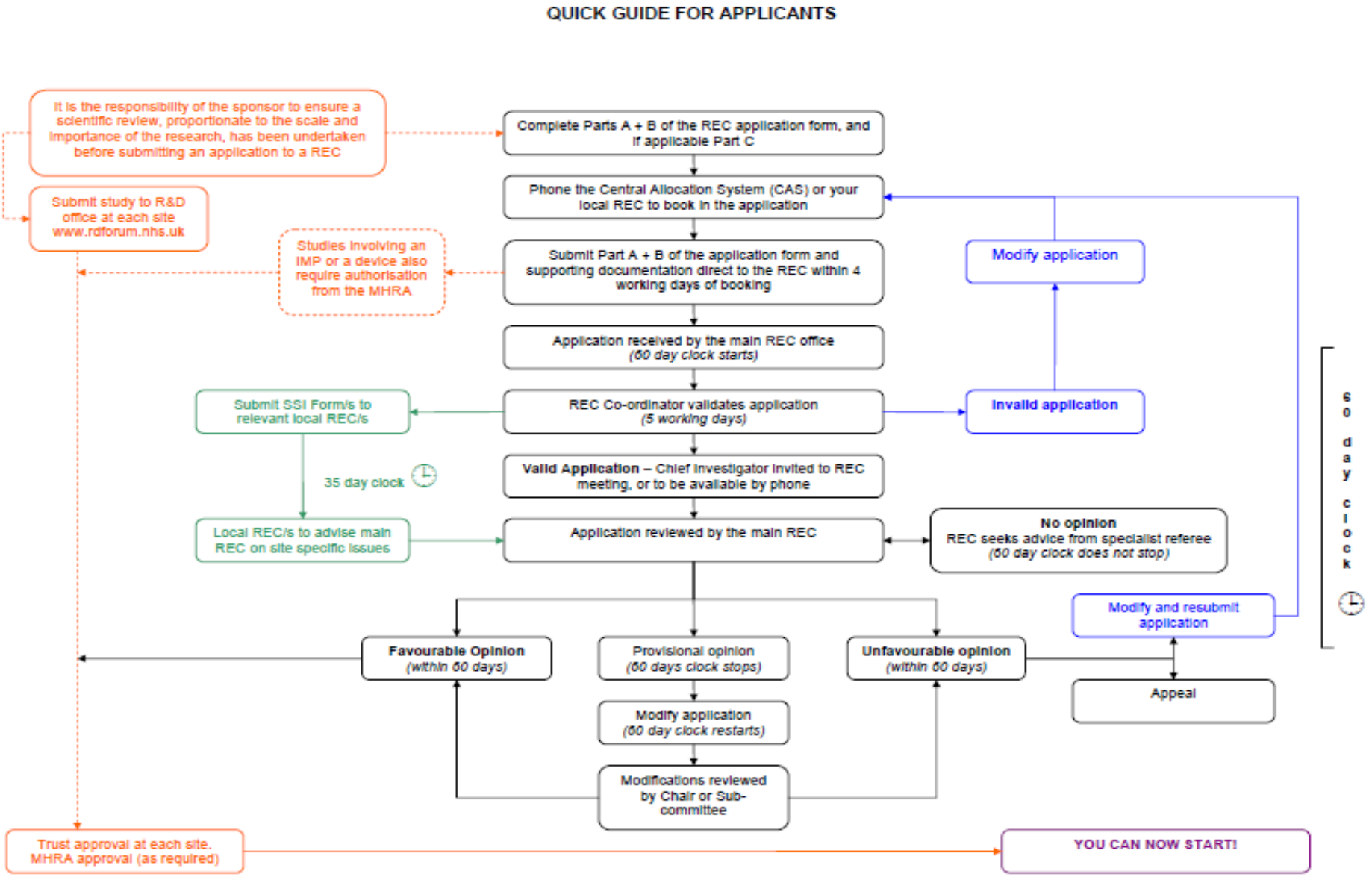


Figure 2.1 CTIMP Application guide

Chapter 3

Pilot Studies

3.1 Reliability of the Compact Fluorescence Spectroscopy and 5-ALA as Photodynamic Detection (PDD) Method in the Oral Cavity

3.1.1 Introduction

Intracellular accumulation of 5-ALA induced PpIX results in increased tissue fluorescence. Differences in the fluorescence intensities between normal and lesion tissue are crucial if the PDD technique is to be a reliable method for detecting malignant change. To that end, reliability is essential.

Initially, intra and inter patients' variation in the fluorescence intensity levels was noticed. In addition, the high fluorescence intensity measurements at some normal anatomical sites such as that of the dorsal tongue further emphasized the need to check the reproducibility of the instrument as a measurement tool in the oral cavity. Hence the following pilot study was undertaken to assess the reproducibility of the technique.

Aim of the study was to detect the reproducibility of the compact fluorescence spectroscopy for measurements taken from the oral mucosa using ALA mouthwash and the detection of fluorescence with the OBS.

3.1.2 Methodology

The study included six patients (PDD004D-9D). The patients were 3 males and 3 females. To confirm the reliability of the photosensitizer and the compact fluorescence spectroscopy as a diagnostic tools used in the detection of fluorescence, the standard

operating procedure was followed and the protocol (explained in the Patients and Methods section) was applied.

3.1.2.1 Data collection

Measurements were taken from 6 patients who were referred to Dundee Dental Hospital/ Unit of Oral Surgery and Medicine clinic, for the diagnosis of suspicious oral lesions. The patients recruited were informed and consented for the procedure of the trial.

Four fixed points were selected from each patient. They were three points taken from well identified anatomical locations of the oral mucosa and one from lesions at any mucosal site:

1- Cheek (right parotid papilla).
2- Tongue (in the middle 2mm away from the tip).
3- Floor of the mouth (mid points between the Wharton's ducts).
4- Lesion sites (one point was chosen from the lesion site).

Two readings from the same site were taken by the same investigator. After making sure the probes were clean and no bubbles of saliva covering the fibreoptic probe surface, the procedure was performed by guiding the probe into the oral cavity and putting the tip over the mucosa with the direction perpendicular to the tissue surface with minimum pressure at the site selected. Then the tissue was exposed to excitation of light at 405nm by pressing the foot control or the (Go) button on the screen of the computer. The spectra were stored on the computer by pressing site

with the same direction to produce (and save) the 2nd reading. The two readings were taken in about 3-5 second time. The saved data were stored as Excel files on the computer and later were grouped as 1st set and 2nd sets of readings for the comparisons.

The total fluorescence emitted measurement system (FEMS) readings were recorded and saved onto the computer for statistical analysis.

3.1.2.2 Data analysis

The measurements at 500nm (autofluorescence) and 635nm (PpIX fluorescence) were obtained from the Excel files and tabulated into 1st and 2nd readings for comparison and analysis to detect the reproducibility of the readings was as follows:

- Comparing the absolute intensity at 500nm of the 1st and 2nd readings.
- Comparing the absolute intensity at 635nm of the 1st and 2nd readings.
- Comparing the fluorescence intensity ratios of the 1st and 2nd readings.
- Comparing the spectral line graphs.

3.1.3 Results

A total of 48 spectra were taken from six patients. The age ranged from 36 to 84 years of age, and the average was 58.5 years. The lesion sites were taken from the case report forms and the intensity peaks of the fluorescence were taken from the Excel files stored on the computer.

The fluorescence intensity peaks of the measured sites were classified as 1st and 2nd readings at 500nm and 635nm as shown in table 3.1.1.

Table 3.1.1. Shows site of the lesions, the absolute fluorescence intensities (FI) at 500nm, 635nm wavelength and the fluorescence intensity ratio (FIR) at 635/500 of the 1st and 2nd readings.

Patient number	Sites of the Readings	FI 500nm		FI 635nm		FIR	
		1st Read	2nd Read	1st Read	2nd Read	1st Read	2nd Read
1 PDD04D	Buccal mucosa	141.2107	141.2113	28.79367	28.792	0.20390572	0.203893
	Tongue	4.16633	4.165	328.923	328.921	78.947899	78.97263
	Floor of the mouth	52.74933	52.74267	163.1737	163.1727	3.09337957	3.093751
	Lesion site	81.48433	81.461	195.823	195.7837	2.40319826	2.403404
2 PDD05D	Buccal mucosa	76.409	76.402	20.71433	20.71367	0.27109804	0.271114
	Tongue	317.6247	317.6243	321.4743	321.4747	1.01211996	1.012122
	Floor of the mouth	123.8757	123.842	85.17733	85.17533	0.68760322	0.687774
	Lesion site	56.149	56.14133	299.68	299.647	5.33722773	5.337369
3 PDD06D	Buccal mucosa	56.803	56.8	6.177667	6.176333	0.108756	0.108738
	Tongue	67.454	67.28867	48.228	48.497	0.71497613	0.720731
	Floor of the mouth	80.56867	80.55533	10.077	19.07133	0.12507343	0.236748
	Lesion site	29.264	29.29733	90.94267	91.07433	3.10766368	3.108622
4 PDD07D	Buccal mucosa	75.17667	75.17833	32.913	32.90967	0.4378087	0.437755
	Tongue	417.601	418.203	394.33	394.326	0.94427456	0.942906
	Floor of the mouth	294.9277	294.9513	61.66267	61.65833	0.20907724	0.209046
	Lesion site	123.137	123.199	57.175	57.15167	0.46432023	0.463897
5 PDD08D	Buccal mucosa	706.623	706.62	60.51367	60.51267	0.08563784	0.085637
	Tongue	29.7437	29.654	82.28867	82.72933	2.76659158	2.78982
	Floor of the mouth	65.47867	65.476	29.463	29.444	0.44996332	0.449691
	Lesion site	461.1293	461.296	128.4197	128.4863	0.27848957	0.278533
6 PDD09D	Buccal mucosa	66.09	66.08933	28.499967	28.49767	0.43122964	0.431199
	Tongue	28.88233	28.786	53.926	54.958	1.86709313	1.909192
	Floor of the mouth	23.94433	24.275	14.441	14.393	0.60310729	0.592915
	Lesion site	42.35367	42.387	23.45767	23.312	0.55385212	0.54998

3.1.3.1 Comparison at 500nm

A comparison between the 1st reading and the 2nd reading at 500nm was ploted in the tables. The summery table shows similar distribution of the data in relation to the mean, median, inter quartile range and standard deviation. However the means were deviated from the medians which represent skewed non normal distribution.

Group	No.of Readings	Mean	Median	25%	75%	Std Dev
1st reading (500nm)	24	142.619	71.315	47.552	132.543	173.010
2nd reading (500nm)	24	142.652	71.233	47.565	132.527	173.064

Table 3.1.2. Summary statistics of the 1st and 2nd readings at 500nm.

The difference in the median values between the two groups using Mann-Whitney U Statistic= 285.000 284.000, T = 591.000 (P = 0.959). This indicates there was no statistically significant difference (p>0.05) between the 1st and 2nd readings at 500nm wavelength.

Figure 3.1.1 shows the 1st fluorescence intensity of the 1st reading in comparison with the 2nd reading at 500nm. The bars were equal in values for both readings in spite of the difference in the values which were related to the sites that obtained from. Sites 10 and 22 showed very slight differences between the 1st and 2nd readings.

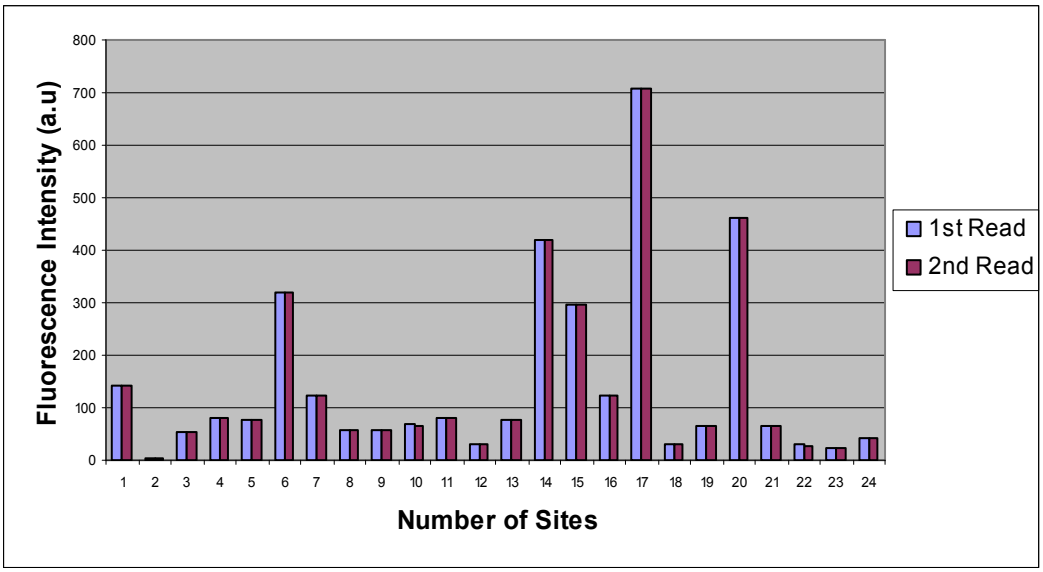


Figure 3.1.1. Comparison of the 1st and 2nd readings at 500nm wavelength.

3.1.3.2 Comparison at 635nm

The same method of analysis was applied for comparison between the 1st and 2nd readings at 635nm wavelength since the data were obtained from the same spectra. The summary table shows the data description (table 3.1.3).

Group	No of Readings	Mean	Median	25%	75%	Std Dev
1st Reading (635nm)	24	106.928	58.844	28.647	145.797	115.548
2nd Reading (635nm)	24	107.370	58.832	28.645	145.830	115.207

Table 3.1.3. Summary statistics of the 1st and 2nd readings at 635nm.

The difference in the median values between the two groups using Mann-Whitney U Statistic= 284.000), T = 592.000, (P = 0.942), there was no statistically

significant difference (p>0.05) between the 1st and 2nd readings at 635nm wavelength.

Figure 3.1.2 shows the 1st fluorescence intensity of the 1st reading in comparison with the 2nd reading at 635nm. There were some minor differences in bars 4, 11 and 15; however the other bars were equal in value.

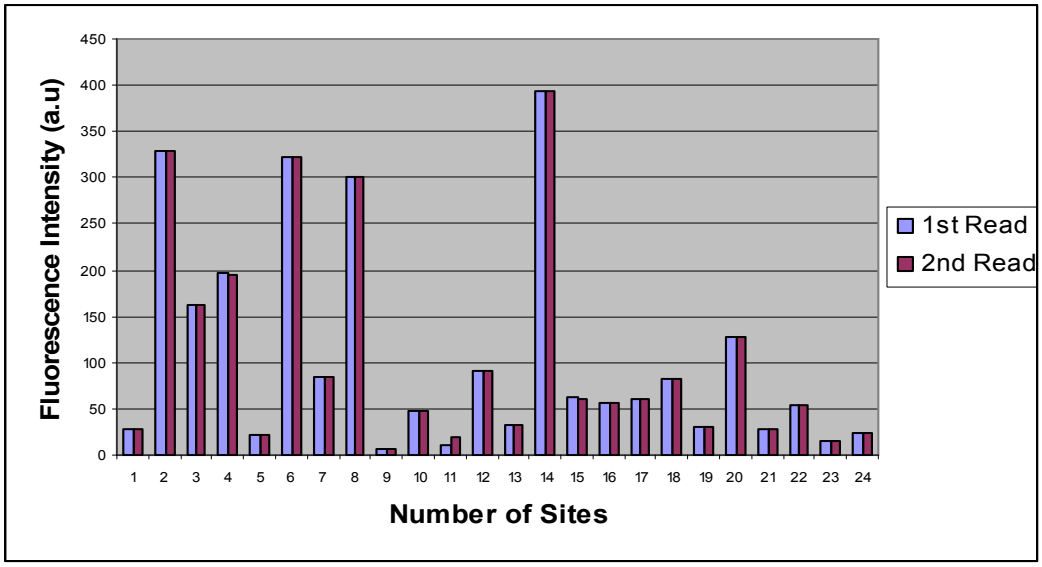


Figure 3.1.2. Comparison of the 1st and 2nd readings at 635nm wavelength.

3.1.3.3 Comparing the Fluorescence intensity ratios

The fluorescence intensity ratio (FIR or red green ratio) could be obtained by dividing the value of the fluorescence intensities at 635/500nm wavelengths. Table 3.1 shows the values of each variable. The FIR of the 1st and 2nd readings' mean, median, inter quartile range and standard deviation is shown in table 3.1.4.

Group	No.of Readings	Mean	Median	25%	75%	Std Dev
1st reading	24	4.379	0.578	0.275	2.135	15.937
2nd reading	24	4.387	0.571	0.275	2.156	15.940

Table 3.1.4. Summary statistics of the 1st and 2nd readings FIR values.

The difference in the median values between the 1st and 2nd FIR values was T = 585.000 using Mann-Whitney U statistic= 291.000 (P = 0.959). There was no significant difference between the FIR of the 1st and 2nd readings.

The data (FIR) distribution (maximum, minimum, median and inter quartile range) of the 1st and 2nd readings were shown in figure 3.1.3.

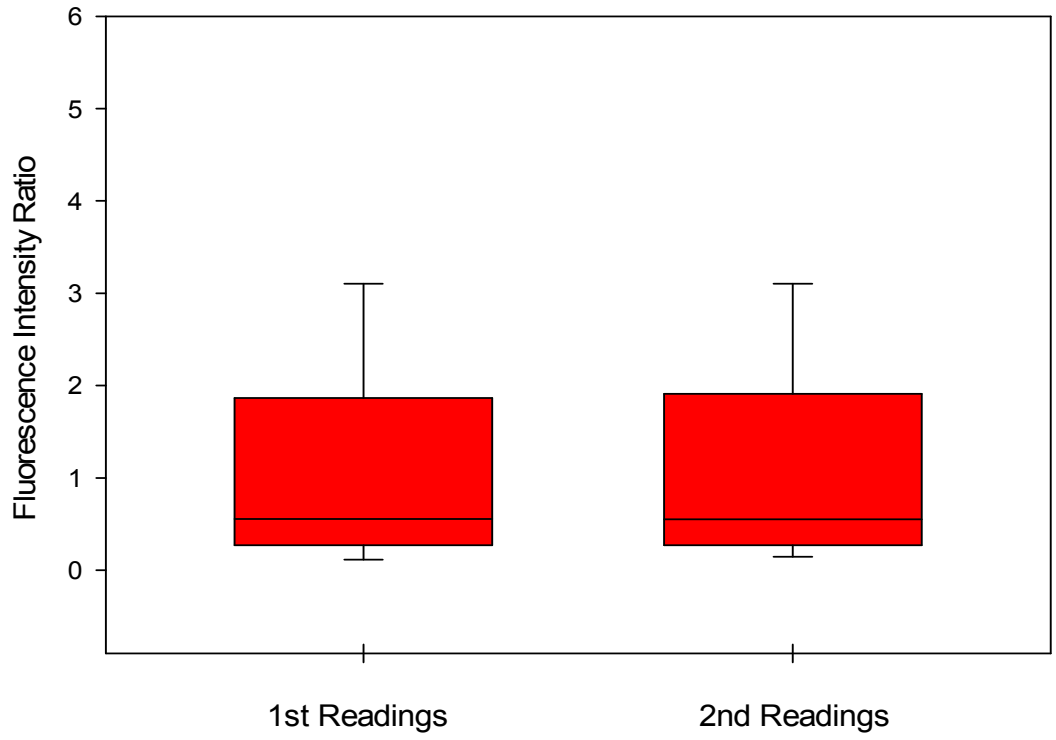
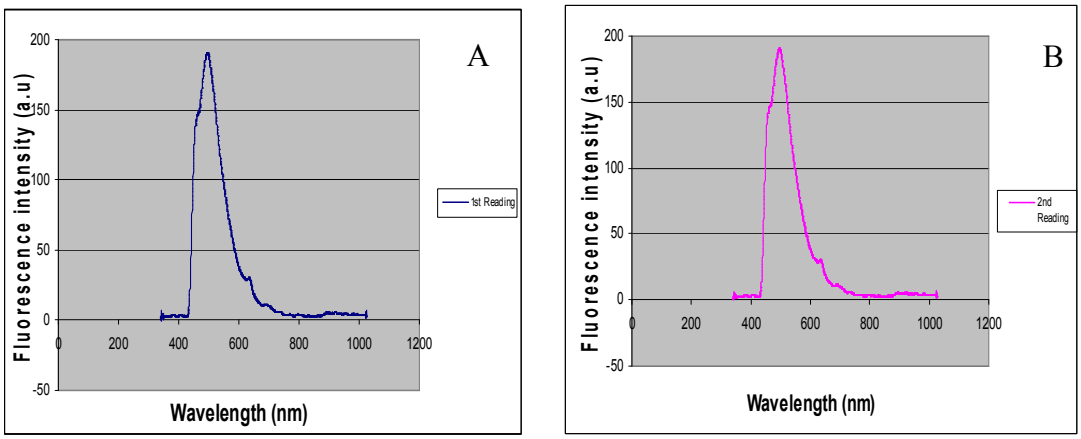


Figure 3.1.3. Comparison of the FIR between 1st and 2nd readings.

3.1.3.4 Comparing the spectral line graphs

The spectra obtained from six patients were averaged in relation to location to show the variation at each site and to detect the reliability of the compact fluorescence spectroscopy in taking the 1st and 2nd measurements. Spectrally the line graphs of the two readings at each site showed identical distribution which indicate there was no variation in the readings obtained from the same site whether the measurement was taken from the lesion or normal locations as shown in the following figures (3.1.4-7).

Figure 3.1.4. Average of the six readings obtained from the normal buccal mucosa. A -1st readings, B-2nd readings, C-combined 1st and 2nd readings.



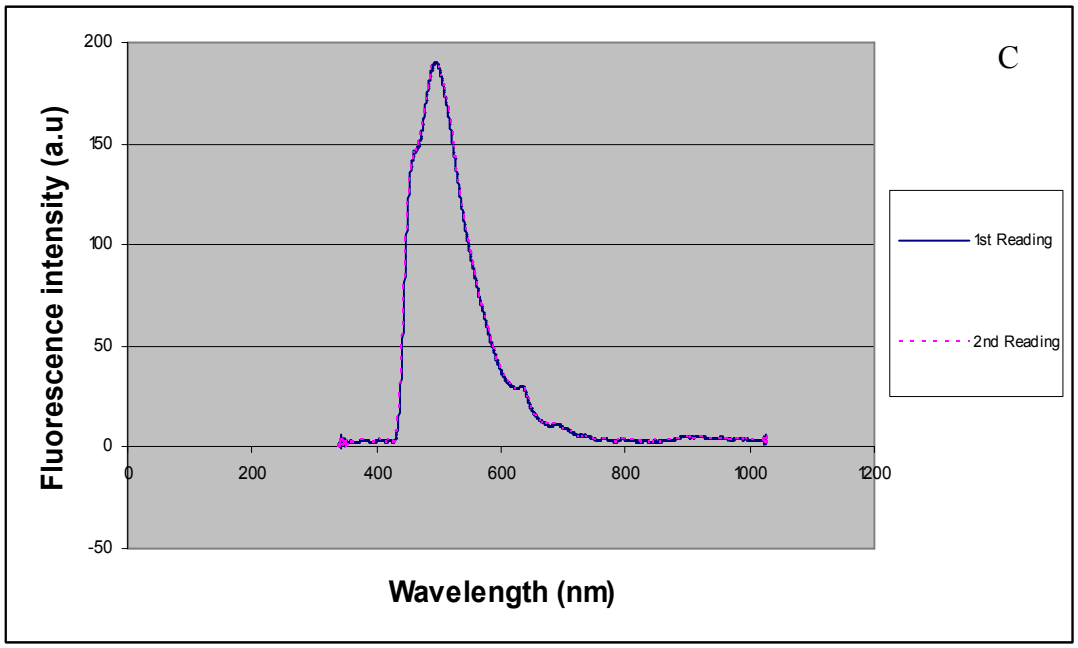


Figure 3.1.5. Average of the six readings obtained from the normal floor of the mouth. A-1st readings, B-2nd readings, C-combined 1st and 2nd readings.

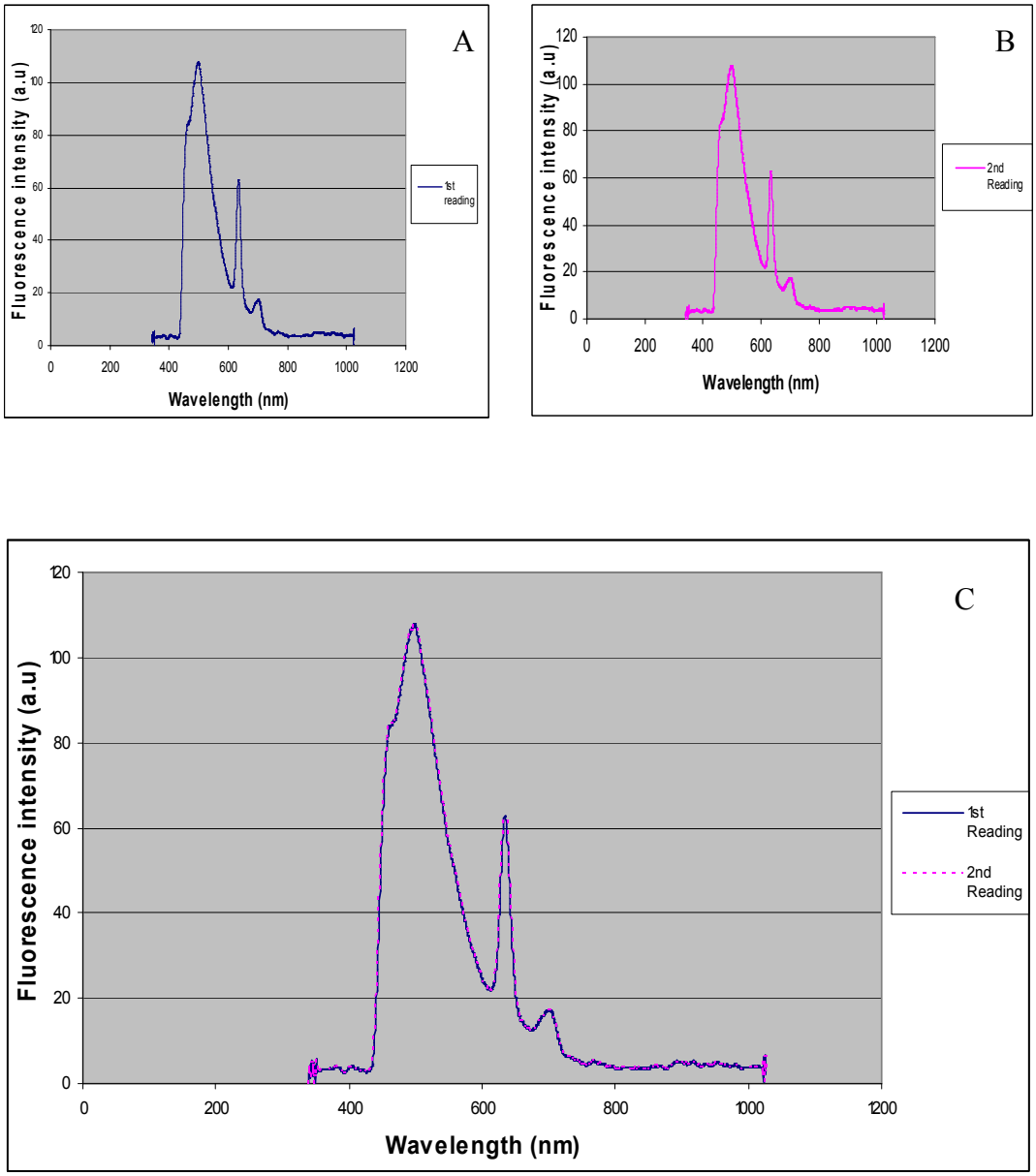


Figure 3.1.6. Average of the six readings obtained from the normal tongue sites. A-1st readings, B-2nd readings, C-combined 1st and 2nd readings.

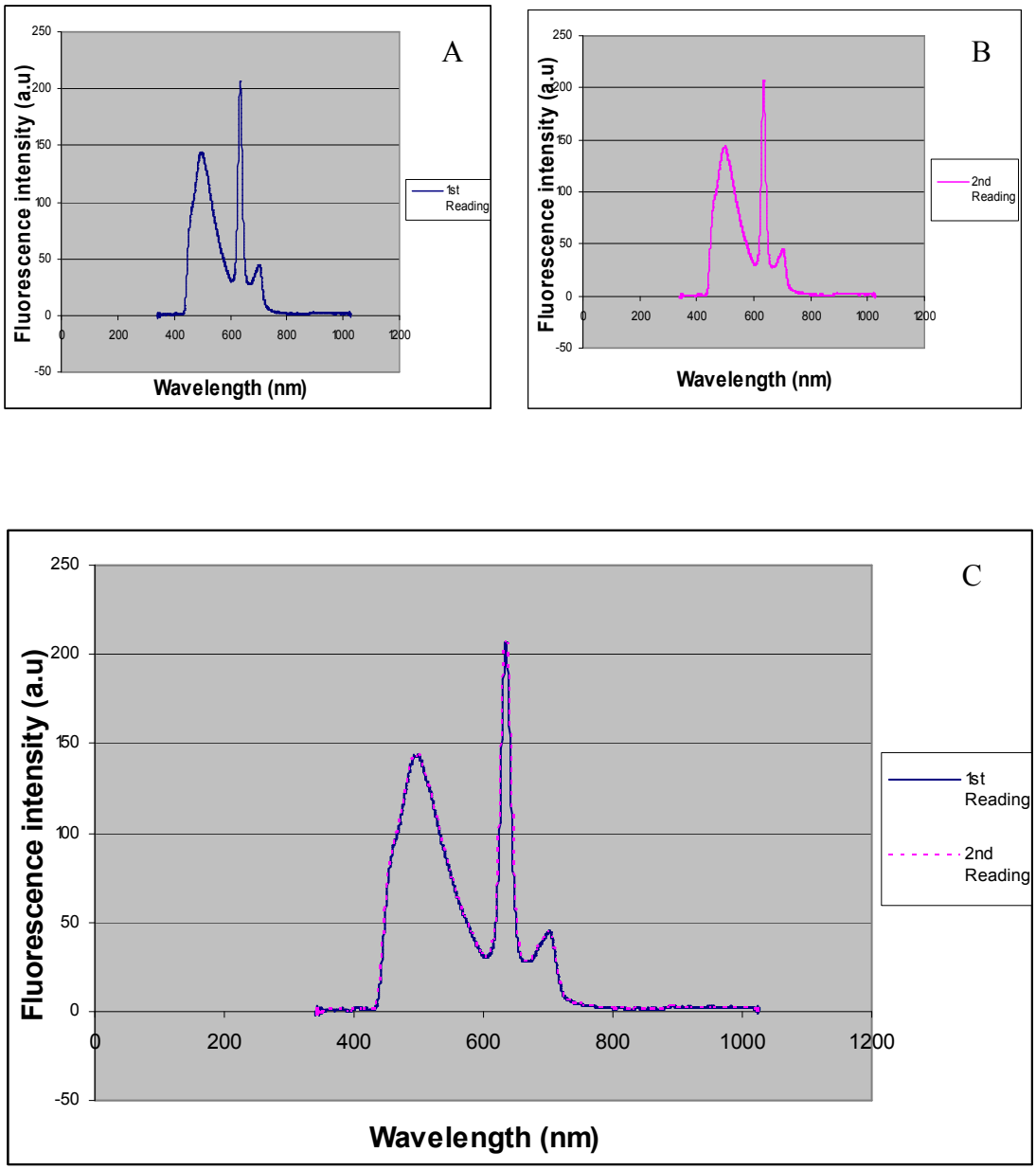
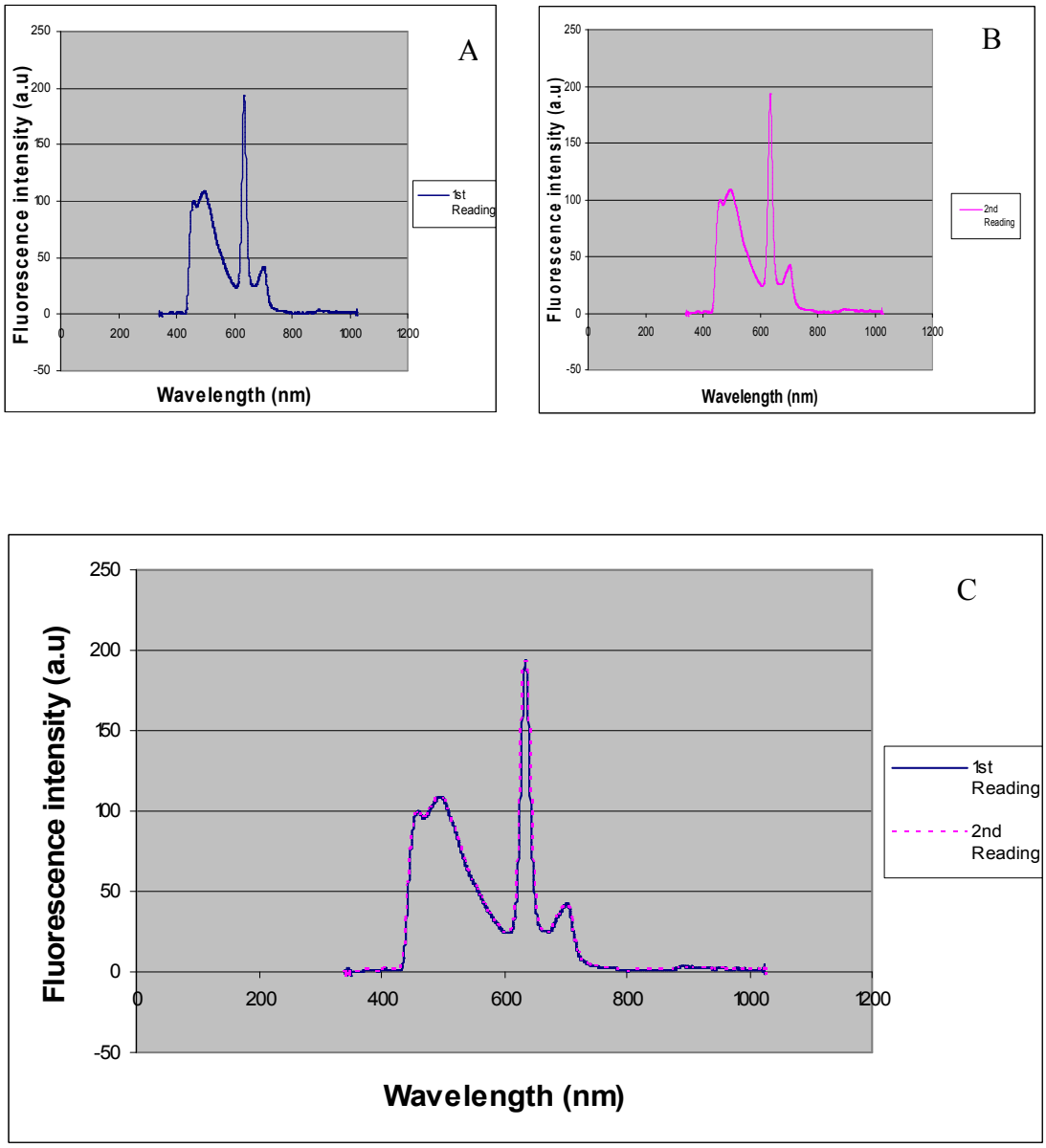


Figure 3.1.7. Average of the six readings obtained from the lesions at different oral sites. A-1st readings, B-2nd readings, C-combined 1st and 2nd readings.



3.1.4 Discussion

The instrument was used to detect skin fluorescence at the photobiology and dermatology unit at Ninewells hospital. Marked inter- and intra-subject variation in ALA-induced PpIX fluorescence occurs in normal human skin. ALA application time, body site and the state of the stratum corneum are all determinants of PpIX fluorescence within subjects and these factors need to be taken into account (Ibbotson *et al,* 2006). This instrument was also used in a pilot study for photodynamic detection of oral cancer, in which further studies was recommended (O'Dwyer *et al,* 2008). In clinical application, inter- and intra-subject variation at the oral sites was noticed which led to a decision of check the reproducibility of this instrument in intra oral application.

Reliability and reproducibility of the method tools needed to be checked carefully. The null hypothesis was that there was no difference between reading 1 and reading 2. All the standard measures were to assess the reproducibility of the technique based upon 5-ALA mouth rinse and the OBS system (compact fluorescence spectroscopy) applied in the oral cavity. The clinical protocol (including darkness of the operating room) was applied to all participants (PDD004-9). One regard to the cleanness of the probe (from air bubbles of saliva) direction of application (perpendicular to the tissue surface) and gentle pressure applied to the oral mucosa were all taken in account (since these factors may alter the light emitted by the probe and light collected from the tissues).

In a study using autofluorescence spectroscopy, de Veld *et al,* found that total fluorescence intensities were highly inconsistent, both intra- and inter-individually

(de Veld *et al,* 2003). The use of ratios of emission peaks at a single wavelength was not investigated in their study. However, they anticipated that ratio-techniques and statistical methods applied to normalized spectra would be more accurate than techniques that rely on the absolute intensity for their diagnostic ability. These techniques would reduce inter-patient variability as well as reproducibility, since spectral shape seems more consistent than fluorescence intensity.

Light emitted from the probe of the instrument used in this project was at 405nm wavelength. It has been reported that using 405nm excitation wavelength, fluorescence emitted from endogenous fluorophores such as collagen, elastin, NADH and the oxidized form of flavin adenine dinucleotide are located in the green spectrum (Catherine *et al*, 2009). This instrument has been programmed to detect the autofluorescence at 500nm.

The principal of 5-ALA induced PpIX is that in excess it results in accumulation of intracellular porphyrins or especially of PpIX which increases tissue fluorescence. Subsequent irradiation of the lesion with visible light matching the main absorption peak of PpIX (405nm) leads to red fluorescence emitting from PpIX peaking at (635nm).

The absolute fluorescence peaks of each reading in this pilot study were recorded at 500nm and 635nm scale of the X axis of the line graphs that displayed on the screen of the instrument.

The statistical analysis showed very similar distribution of the data both at 500nm and 635nm for each site. In spite of the fact that the comparison of the absolute fluorescence intensities of both wavelengths as a source of the fluorescence intensity ratio (FIR) measurements would be enough to check the reliability of the method, it was considered worthwhile comparing the FIR of the 1st and 2nd readings, since the FIR value more accurately expresses the biochemical and structural changes of the cells. This value will be used for the results of this research. In addition, it was noted that the identical shapes of the 1st and 2nd spectral line graph readings that ranged between 430-730nm as shown in the result figures, would represent the distribution of fluorescence emitted from the natural fluorophores (autofluorescence) and enhanced fluorescence (drug or PpIX fluorescence) of the tissue cells excited by 405nm wavelengths would support the result analysis.

Numerically and graphically the results were closely related. The absolute fluorescence intensities of the 500nm and 635nm which represented the values depicted on the bar graphs were nearly identical. Since the FIR values of the two readings were similarly distributed regarding the minimum, maximum, medians, and inter quartile range and there was no significant difference between the two readings, this result supported the reliability and reproducibility of the use of the instrument in intraoral measurements using ALA mouthwash. The null hypothesis was confirmed. There was no significant difference between reading 1 and reading 2.

3.2 Determination of the Elevated Red Spectrum (635nm or PpIX) Fluorescence Peak of the Tongue Mucosa

3.2.1 Introduction

Tongue is a muscular organ located on the floor of the mouth and covered with specialised mucosa. The mucosa covering the tongue is thick and keratinised with papillae projecting from the surface. The mucosa is different from the other oral coatings due to the presence of the various forms of papillae and taste buds, and thus called specialised mucosa. The tongue coating is sensitive and in many instances might be altered duo to local or systemic factors. These features make it unique when considering lesion diagnosis and application of adjunctive diagnostic techniques (e.g. toluidine blue).

After oral topical application of the photosensitiser (5-ALA), a fluorescence glow was primarily noticed when using the Wood's lamp. Spectrally, the spectroscopic line graphs of the tongue surface mucosa (Photodynamic Detection method) showed elevated red (635nm) peaks, therefore the FIR measurements were increased in comparison with other normal oral mucosal sites (leading to a false positive in respect of potential dysplasia).

Aim of the study was to understand why there was elevated red (635nm) fluorescence peaks from the normal dorsal surface of the tongue; is it due to drug trapped in superficial cells, and/or bacterial reduction of ALA to PpIX?

3.2.2 Methodology

Four volunteers participated in this experimental study. The trial was conducted in the photobiology unit at Ninewells hospital. The application of the trial was to some extent consistent with the protocol regarding preparation of drug and method of the spectroscopic measurements described in the Patients, Materials and Method section.

One 5-ALA vial of 1.5g was dissolved in 50ml distilled water and divided into four equal amounts. The concentration of the drug was the same as that prepared for the patients recruited for the trial. Each participant had used 12.5ml of the prepared drug and held it in his/her mouth for 15 minutes. After rinsing with the drug and reminding participant not to eat, drink or smoke, 90 minutes later the OBS reading were obtained from the dorsal tongue for the participant in a dark room, The tongue was scraped with a Cytobrush (MedScand, Colgate Medical Ltd, England) for one participant and the wood spatula used for the other 3 participants. The collected coatings were kept in the freezer for the later HPLC (high pressure liquid chromatography) analysis. The method applied was to quantify compounds present in the dorsal tongue scraped material and to detect porphyrin levels as an indication of whether the fluorescence is due to PpIX within bacterial or superficial tissues (bleeding was not obtained).

Samples of whole saliva were collected in plastic tube to detect protoporphyrin levels as control to the HPLC result analysis.

The second OBS readings were taken after scraping the tongue surface from the same site as the previous (1st) reading for data comparison for pre and post tongue scraping fluorescence (FIR or red/green ratio) analysis.

Furthermore, for one participant readings were taken from the contra lateral side of the dorsal tongue before and after using 2%chlorhexidine (disinfectant) mouthwash. The tongue scraped material was collected using a plastic spatula for HPLC result analysis.

3.2.3 Results

Few samples of tongue scraped material were collected for HPLC analysis. A total of 8 spectral (readings) were collected. These were 4 pre scraping and four post scraping spectra. The absolute fluorescence intensity peaks at 500nm, 635nm and their ratios (635/500nm) of pre-scraping and post-scraping measurements are shown in table 3.2.1. The level of pre-scraping and post-scraping fluorescence intensity ratios is shown in figure 3.2.1. The post-scraping FIR was increased in participants 1 and 4, participant 2 showed reduction in FIR, however participant 3 showed no difference in post-scraping FIR.

Participant	Pre scraping			Post scraping		
	500nm	635nm	Ratio	500nm	635nm	Ratio
1	218.934	453.1167	2.0696	217.6997	546.743	2.511
2	24.20367	25.924	1.071	26.42067	22.47467	0.85
3	227.7123	668.3893	2.9352	227.7123	668.3893	2.9352
4	191.0317	440.344	2.305	110.3267	448.4667	4.065

Table 3.2.1. Pre and post-scraping fluorescence intensities at 500nm and 635nm readings and red/green ratios of the tongue.

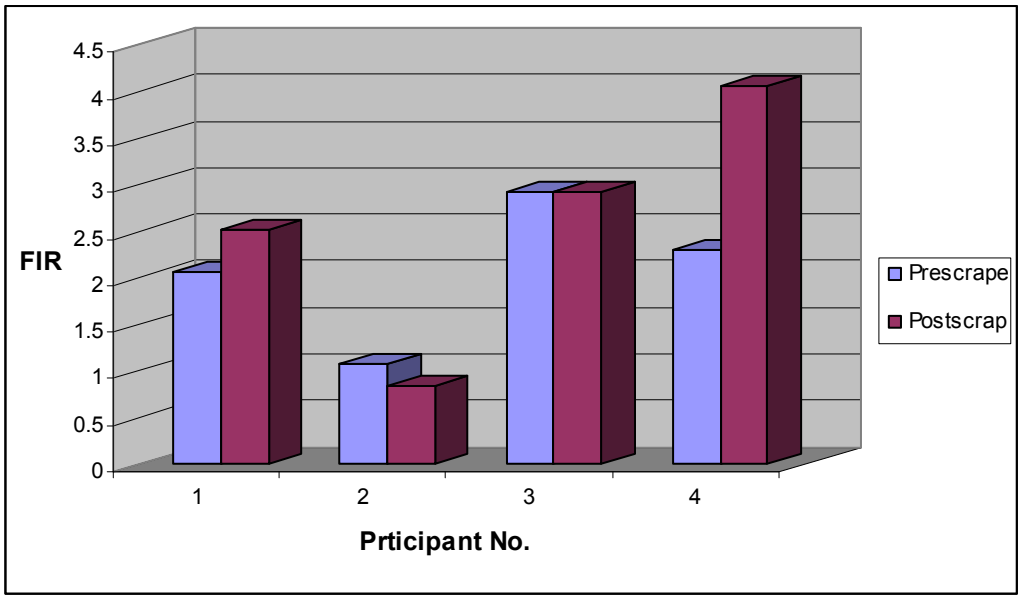


Figure 3.2.1. Pre and post scraping fluorescence intensity ratio or red/green ratio of four participants' tongue.

From the contra lateral side of the tongue of one participant (volunteer 4), one reading was obtained, then chlorhexidine mouth wash was applied and the 2nd reading was obtained for the comparison i.e. two extra spectra were obtained from one participant one before and one after application of chlorhexidine. The fluorescence intensity measurements were summarised in table 3.2.2. There was reduction in the FIR (635/500nm) measurement in post scraping site after chlorhexidine mouth rinse application was noticed as shown in figure 3.2.2.

Participant	Pre scraping			Post scraping		
	500nm	635nm	Ratio	500nm	635nm	Ratio
1	154.8657	594.9307	3.841	172.683	476.877	2.761

Table 3.2.2. Pre and post scraping fluorescence intensities at 500nm and 635nm readings and red/green ratios of the tongue after chlorhexidine mouthwash.

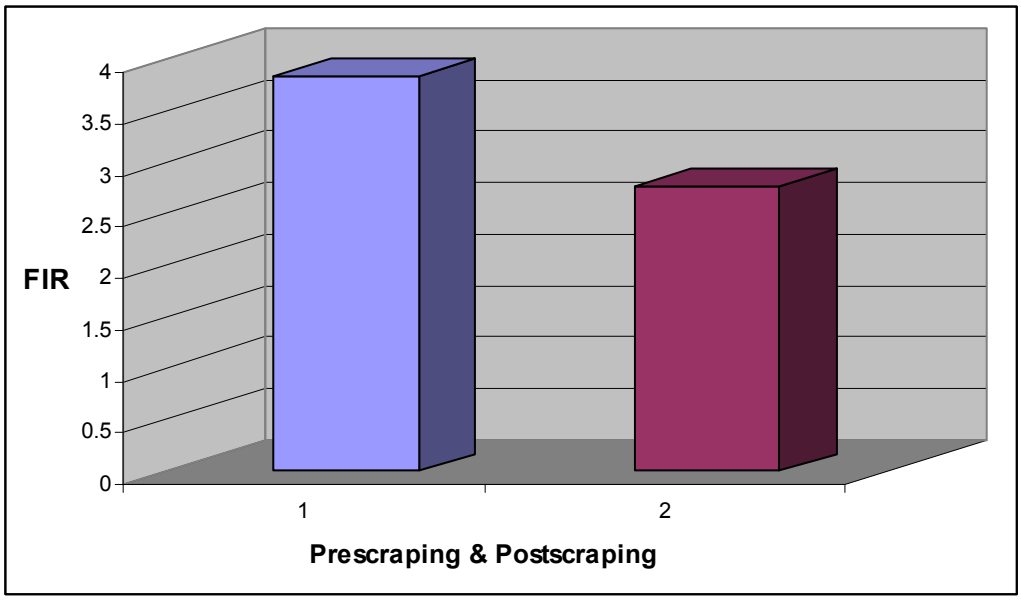


Figure 3.2.2. Pre and post scraping fluorescence intensities ratio (635/500nm) or red/green ratio of the tongue after chlorhexidine mouthwash.

HPLC analysis

Using HPLC, Protoporphyrin was detected in only one sample of the tongue scrape. The level of Protoporphyrin in the saliva of the participant was 5.4 +/- 0.4 % and in the tongue scrape was 5.5%. However the other samples were not analysed because the amounts were below the level of quantifications.

The 2nd sample of tongue tissue was obtained from one participant who used 2% chlorhexidine mouth wash after 5-ALA mouth rinse. The level of Protoporphyrin in the saliva was 33.8% while in the tongue scrape was 71.0%.

3.2.4 Discussion

In reviewing the literature, previous studies concluded that different tissues have different pharmacokinetic responses in the rate they syntheses protoporphyrin, and its conversion to heme products (Vaidyanathan *et al*, 2000). In human subjects, ALA-induced protoporphyrin IX (PpIX) fluorescence images from the normal tongue were compared to images obtained from patients with squamous cell carcinoma (SCC) of the tongue (Thong *et al,* 2007). Tongue mucosa showed red fluorescence at 635nm using autofluorescence spectroscopy (de Veld *et al*, 2003).

Our observation of the tongue glowing with the Wood's lamp (and the OBS detection if fluorescence at 635nm) and different spectra line-graphs readings motivated the investigators to try to understand why the normal dorsal tongue was fluorescing. Prior to conducting a clinical trial a pilot study was undertaken to see if it would be worth submitting a more formal request for a full clinical investigation. The results of this preliminary study were contradictory.

The volunteers participated in this pilot study were investigators and photobiology unit staff members.

Collection of tongue scrapings were tried using the Cytobrush, wooden tongue blade and plastic blade since the investigators were unsure which one was the best method to collect samples of dorsal tongue for HPLC protoporphyrine detection.

The readings in one volunteer (2^{nd}) showed slight reduction in the tongue fluorescence intensity ratio (FIR), while in another volunteer (3^{rd}) there was no change in the FIR readings; however there was post scraping increase in the FIR of

two volunteers (1st and 4th). The result was not conclusive but the variation could be attributed to the method of scraping which was not standardised since the method of collecting tongue sample using the Cytobrush was different from that of the tongue blade i.e. the post application bacterial collection and/or mucosal irritation might have influenced the post scraping fluorescence measurements.

Unfortunately the Cytobrush and plastic spatula were the only methods that could collect samples of superficial tongue tissue, while the wooden tongue blades were not useful since the liquid was absorbed by the wood so the samples could not be detected and analysed by the HPLC to quantify the protoporphyrin level. HPLC was able to detect and quantify the Protoporphyrin percentage present in the saliva samples as control.

Protoporphyrin (as a precursor of heme) accumulates in cells of all living organisms, such as erythrocytes and bacteria (Thunell, 2000; Latunde-Dada, 2009). Detection of this element represents the level of the cellular presence and activity in the samples. Saliva, as well as the tongue scrape, showed evidence of Protoporphyrin however there was no difference in the amount present in both samples (saliva and tongue scrape) collected from the participant.

There was a difference in the protoporphyrin percentage between the tongue scrape tissue and the saliva in the samples obtained from the pre and post chlorhexidine mouth rinse application. This might be due to the effect of chlorhexidine on the bacteria in the saliva (flora) if the bacteria contained PpIX i.e. the chlorhexidine removed some PpIX positive bacteria.

There was a slight reduction in the pre and post scrape FIR measurements taken from the dorsal tongue which could be due to reduction of the red (635nm or PpIX) fluorescence peak resulted from the removal of the superficial layer of the tongue coating that presumably contain living cells and microorganisms that contained PpIX. A previous study using the Cytobrush to obtain oral cell failed to detect basal cell (Ogden *et al,* 1991) hence we can be confident that the tongue scrapings were of superficial dead cell and not actively dividing cells that could have taken up and metabolized the ALA to PpIX intracellularly.

The sample size was too small and there was inadequate evidence to conclude why the tongue FIR measurements were higher in comparison with other normal mucosal sites, although there was a suggestion that oral bacteria were responsible.

Chapter 4

Patients, Materials and Methods

4.1 Instruments

4.1.1 The Compact Fluorescence Spectroscopy or Optical Biopsy System (OBS)

The instrument used for this investigation was a computer based system composed of spectrometer, cables (connection, control and fibre-optic) and laptop downloaded with software to analyse the spectrums of light emitted from the tissue for the result analysis as shown in figure 4.1.

The instrument has previously been used in a number of trials conducted at Ninewells hospital to detect the lesions in various organ systems both for the photodynamic detection and photodynamic therapy. The results have been published in scientific journals (Nadeau *et al,* 2002; Nadeau *et al,* 2004; Ibbotson *et al,* 2006; O'Dwyer *et al*, 2008; Eljamel *et al,* 2008; Lesar *et al*, 2009).

A detailed description of the compact fluorescence spectroscopic tool and graphs were detailed in the review of literatures section.

The system schematic diagram of the experimental set-up is shown in figure 4.2. The system incorporates a 5 mW GaN diode laser, emitting at 405 nm, the wavelength at which the highest absorption peak of the PpIX spectrum occurs. This laser is coupled via a 600 μm core optical fibre into an electronic shutter, which also contains a blocking filter to prevent small amounts of 635 nm light emitted by the GaN laser from reaching the oral mucosa. The electronic shutter ensures that the oral mucosa is only exposed to light from the laser for the duration of the measurement.

Due to the high efficiency of the optical design, a 1 second exposure to light at an intensity of 0.5 mW/cm^2 is sufficient to produce measurable fluorescence spectra. A bifurcated fibre couples this excitation light to the oral mucosa and the fluorescence to a compact spectrometer for spectral analysis. The spectrometer, preceded by a 455 nm long-pass filter to remove the backscattered excitation light, has a resolution of 10 nm and wavelength range of 430 to 730 nm. All aspects of the instrument's operation are controlled using a laptop PC and custom written software. The acquired spectra are analysed using pre-recorded spectra and a least squares fitting routine to obtain the relative strengths of the background optical fibre fluorescence, tissue autofluorescence, PpIX fluorescence and photoproduct fluorescence. These various spectra are shown in Figure 4.3, as they would appear in a fluorescence spectra from a measurement made on subjects to whom 5-ALA has been administered (heavy dark line). The spectral components due to the other fluorescence signals are shown by the lighter grey lines (figure 4.3).

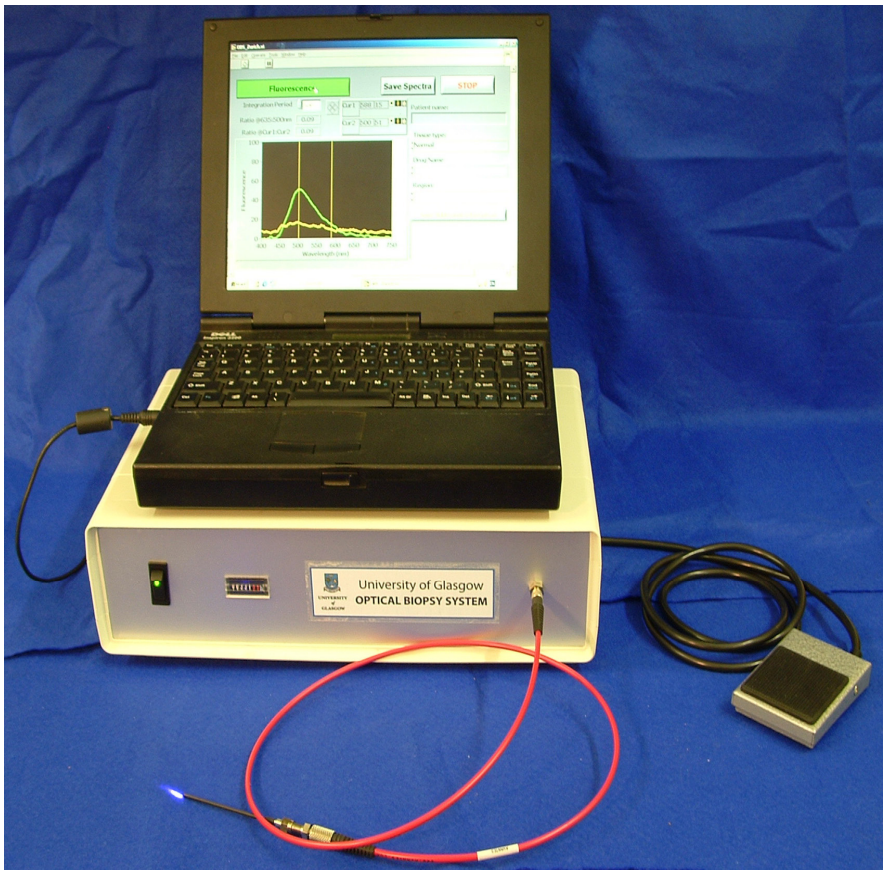


Figure 4.1. Photograph of the Optical biopsy system (OBS).

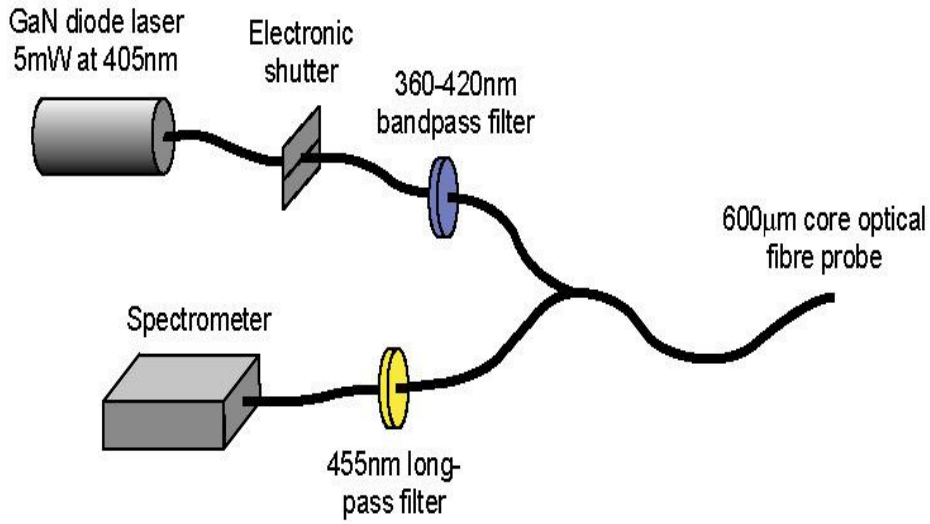


Figure 4.2. Schematic of the OBS used to perform the measurements (Nadeau *et al,* 2004).

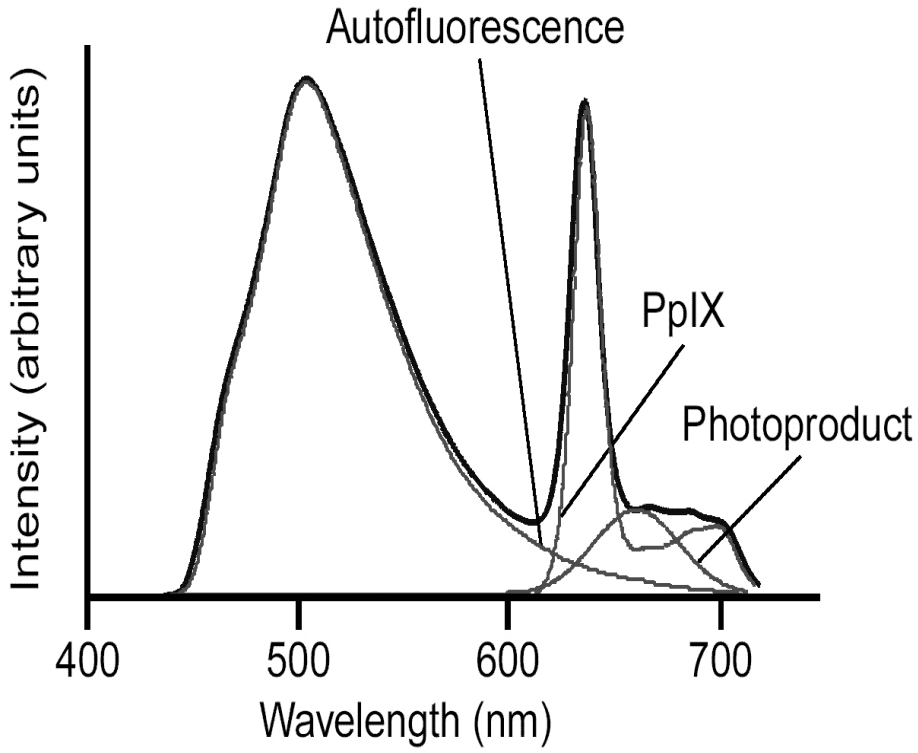


Figure 4.3. Graph of the overall fluorescence measured (dark line), and its components (grey lines) (Nadeau *et al,* 2004).

4.1.1.1 Probe of the system

We have used the probes with different lengths developed to be used for the oral cavity readings Figure 4.4. The width is of 2mm in diameter and consists of optic fibre for illumination and fluorescence detection. The probe emits light transferred from the system cable and deliver 405nm excitation wavelength since results from previous studies suggested that this wavelength is located within the range of wavelengths which produce the greatest discrimination between normal and abnormal tissue (Vengadesan et al, 1998; Eker et al, 2001). The probe also collects the fluorescence emitted from the tissue to be transferred to the spectrometer through the system fibre-optic cable.

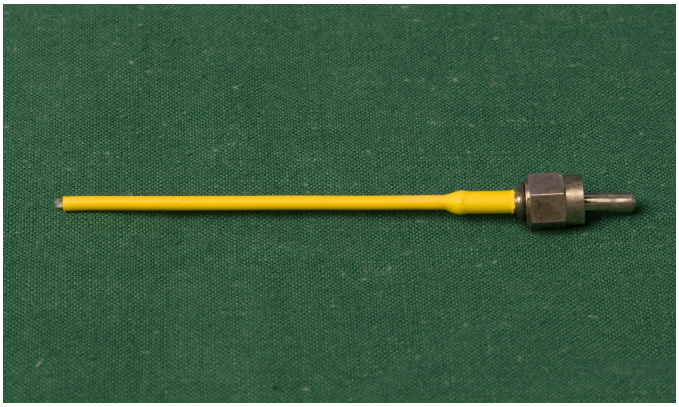


Figure 4.4. Fibre optic probe is connected to the OBS.

The probe is the only component requires sterilization which was sterilized with 100% ethylene oxide (EtO) to a sterility assurance level and the device is packaged in a heat sealed pouch.

The investigator had to fill out the sterilization form at the photodynamic centre/Ninewells hospital and submit the probes soon after finishing the trial since the sterilization requires two weeks for the probes to be used again.

4.1.1.2 How does the OBS work?

After insuring all the cables of the laptop and the instrument were connected together and plugged in the electric source, the instrument was turned on by pressing the green lighted button on the front side of the instrument (Figure 4.1) and turning the laptop on. The laptop was pre-installed with OBS program software designated for this purpose and the software existed as an icon on the screen. A foot paddle connected to the instrument through a cable plugged in the laptop to switch on the laser light for 1 second for tissue illumination, or the alternative when the cable was not available by asking the assistant nurse to press a Go button on the screen of the

laptop. The collected light is analysed in the form of graphs in real time and displayed on the screen of the laptop (Figure 4.1).

The results of the graphs displayed on the screen of the computer, colours of the backgrounds and the fluorescence lines, could be copied from Excel file and transferred to Word and copied as shown on Figure 4.5. The green line represent the fluorescence of the cells due to the presence of the naturally occurring fluorophores and the presence of the photosensitizer drug 5-ALA picked up, metabolized and changed to protoporphyrine IX (PpIX), while the yellow line graph represents the background (BG) scattered light subtracted from the total fluorescence to display the final outcome graphs that finally exist on the screen.

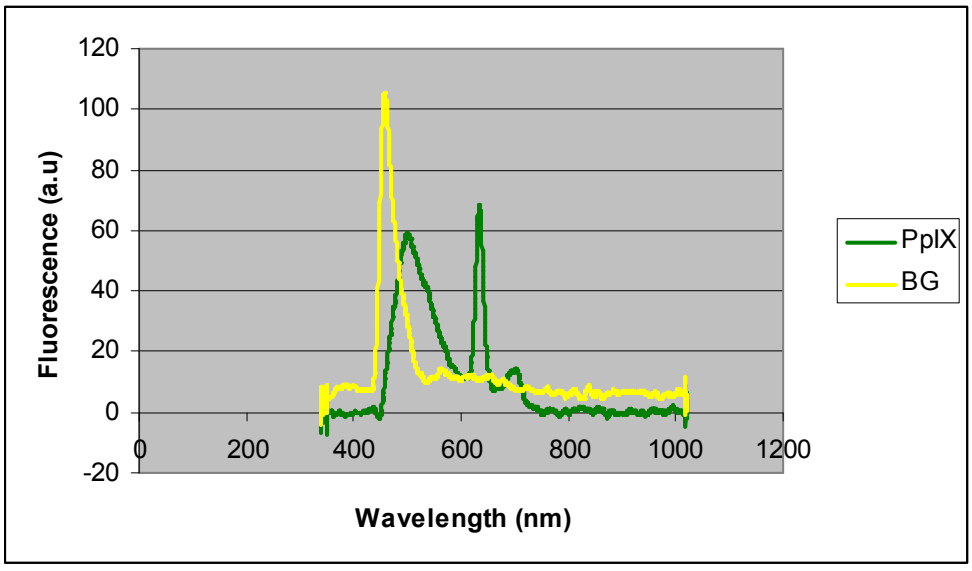


Figure 4.5. Graph of the fluorescence measured as shown in the screen (results) .

4.1.1.3 Recording and saving the data by the OBS

The instrument has the ability to measure the tissue fluorescence at any wavelength ranged between 339nm up to 1000nm. The spectra of light are analysed and saved on the laptop in the form of Excel files. Emission and collection of light from the tissue for 1 second is stored in a form of one Excel file of that site. The file contains three columns; each one consists of 2048 values or numbers. The first column represents the wavelength ranges between 339nm up to1000nm, the second column is the readings of the fluorescence emitted by the tissue and the third column is the readings of the background subtracted from the overall fluorescence reading.

Each wavelength (339nm-1000nm) has been registered three times. This was to analyze three readings of fluorescence and background to draw and display the outcome graph on the screen as shown in Figure 4.5.

On the screen of the laptop there was a slot to show the Fluorescence intensity ratio (FIR) (red/green ratio) or the ratio of the location of the two cursers over the wavelengths required for the investigation at the moment of the reading.

On the right side of the screen, there were slots used to type name of the patient and the diagnosis of the lesions. To comply with the rules of the Ethics in conducting the clinical trials in maintaining and protecting the confidentiality of the patients the slot of the name was typed by the participant ID e.g. (PDD001D, PDD002D….etc) to identify the Excel files, while the other slots were left unidentified.

4.1.2 Short Wavelength Lamps

4.1.2.1 Wood's Lamp

The woods lamp was invented by an American physicist called Robert Wood in (1868-1955). It is now called the "Wood's Light" or "Wood's Lamp". It is a diagnostic tool used in dermatology by which ultraviolet light is shone (at a wavelength of approximately 365 nm) onto the skin of the patient. The clinician then observes any subsequent fluorescence. For example, porphyrins, associated with some skin diseases, will emit pink colour fluorescence. It has many uses, both in distinguishing fluorescent conditions from other conditions, and in locating the precise boundaries of the condition Figure 4.6. a. The wavelength of fluorescence emitted by the woods lamp is further checked by the physicist (Professor Harry Moseley) in Photodynamic Therapy/Dermatology and Photobiology centre at Ninewells hospital and the wavelength fluorescence is shown in Figure 4.6 b. The instrument was used to identify the oral lesions boundaries and glowing areas of the lesion to be measured by the OBS.

4.1.2.2 Torch Lamp

The device was developed by the University of Dundee/ Electronic engineering department (PhD student). The lamp uses Led chip material based on GaN ultra violet light. The wavelength emitted is 395nm as shown in Figure 4.7. a&b. The device has a switch to change the current from 20 to 30mA and gives different intensities of light. The handle of the probe is small and adjustable so could be used to access the tiny lesions at distant sites intra orally. The instrument was used when received and started the application in PDD09D and lasted in PDD27D. The data of 20mA was collected

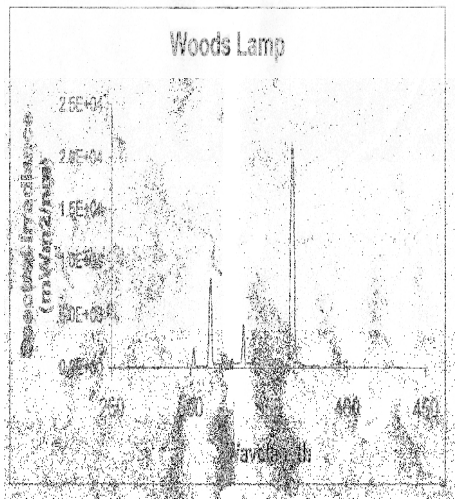
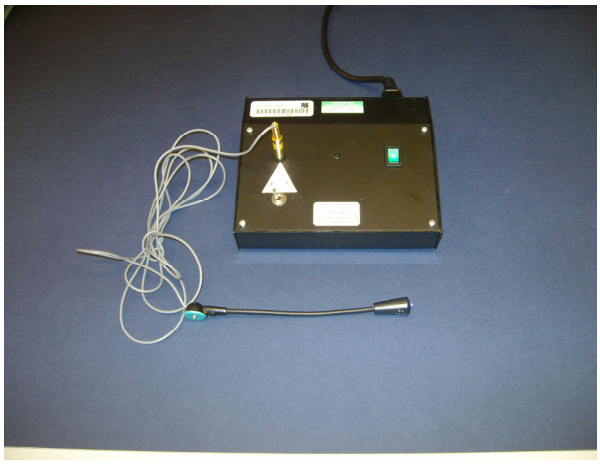


Figure 4.6. a. Wood's lamp is shone 365nm wavelength b. Wavelength emitted by the lamp.



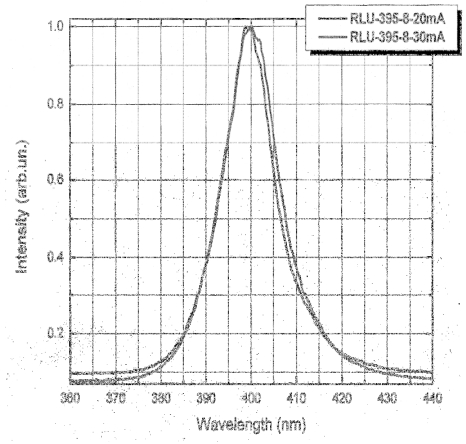


Figure 4.7. a. The torch lamp is shone 395nm wavelength. b. Intensity of fluorescence emitted by the lamp at different

since 30mA was upgraded in the middle of the trial.

4.2 The Mouth Rinses

4.2.1 Chlorhexidine

The mouth rinse was used before the use of the photosensitizer to disinfect the bacterial flora of the oral cavity. 0.2% concentration chlorhexidine gluconate is a bacteriostatic chemical that kills both gram-positive and gram-negative microbes although it is less effective with some gram-negative microbes (WHO, 1998). The mouth was bathed with 5ml of chlorhexidine for 2min before the use of 5-ALA mouth rinse

4.2.2 Photosensitizer prodrug (5-ALA)

Vials of 5-aminolevulinic Acid 1.5g were used for this trial. It was supplied by Medac GmbH postfach 30 36 29 D-20312 Hamburg. The generic drug was ordered for preparation as a mouth rinse as per protocol to Ninewells Hospital, Photodynamic Therapy Centre. The Drug was present in clear vials and stored at room temperature in the pharmacy room in a dark cabinet. A digital thermometer monitored the temperature which should not exceed 25C (figure 4.8).

Figure 4.8. Vial of the photosensitizer.

4.3 Patients Recruitment

The steps of the procedure were followed at both sites of the trial (Dundee and Glasgow) in an attempt to standardize the method of the trial. The clinical nurse specialist (Carol Goodman) was assisting the author at the beginning and coordinating the trial in Glasgow, since she had jobs at both sites.

Patients were recruited for three sites, Dundee Dental Hospital/Oral Medicine and Diagnosis Clinic, the Oral and Maxillofacial clinic at Ninewells hospital and patients referred to the maxillofacial clinic at Glasgow Southern General Hospital.

The patient's criteria for recruitment were as follows;

4.3.1 Inclusion Criteria

- Men and women age over 18 years.
- Willing to participate in the trial.
- Oral lesions affecting the mouth lining.
- Able to use the mouthwash for 15 minutes.
- Able to comply with instructions.
- Be able to sign and date a valid consent form prior to entering the trial.

In addition to women were eligible to enter and participate in this study:

- Non-childbearing potential (postmenopausal or physiologically incapable of becoming pregnant e.g. Hysterectomy).
- Child bearing potential and has negative pregnancy test (urine or serum) at screening.

4.3.2 The Exclusion Criteria

- Younger than 18 years.
- Those participants who couldn't give their own informed consent.
- Pregnant women/women pregnancy test will be offered.
- Breast feeding women.
- Patients who have porphyria.
- Patients with known malignancy.

Patients with known malignancy were excluded as designed in the protocol, however, amendments of the protocol were submitted to REC, MHRA and local R&D and approvals were obtained to include oral malignancy patients since few dysplastic lesions were recruited in Dundee and Glasgow were slow in recruitment. The only known cases recruited were the last two cases in Dundee (PDD026D & PDD027D) before the MHRA inspection.

The patient information leaflet (Patient information sheet/Protocol code number CG/7657- Version 5-2007-07-07 see appendix 3 A-C) was given to each patient agreeing to take part in the trial and the procedure was discussed in detail. A consent forms (V4 AUG 07 upgraded to V5 and V6 January 2009 see appendix 2 A&B) were completed and signed by the subject and the authorised person. The original copy of the consent form was kept in the medical or the dental notes, additional copies were kept in the TMF or CIF and for the patient. The consented patients were given enough time before the procedure and informed that they have the right to drop out of the trial at any time.

4.4 The Clinical Procedure

It was planned that the patients would be tested at the Oral & Maxillofacial Unit; Ninewells Hospital as the Optical Biopsy System (OBS) was kept in the Photobiology and Physics laboratory at Ninewells Hospital. However the limited access to the maxillofacial clinic and the constraints we had faced to conduct the trial led us to change the venue to the photodynamic centre then later had to move to the Clinical Research Centre to continue the trial and to reach the target number of patients designated in the protocol.

The patients were asked to come at 15 minute intervals for the mouthwash with at least 2 patients per session since the drug was costly.

On the day of the trial, the patients were examined clinically by the investigator and the Case Report Forms (CRF) (see appendix 4 A-G) filled out with detailed description of all the information required for the documentation of the history, systemic condition and clinical description of the oral lesions.

Each patient participated in the trial was coded PDD---D (e.g. PDD001D, PDD002D….etc) to maintain the confidentiality of the patient and comply with the ICH-GCP and signed by the investigators who were only authorised to conduct the trial.
The case report forms, standard copies designed by the trial investigators at TCTU and the additional forms designed by the author were filled out and signed by the investigator before starting the mouth rinse and taking the intraoral measurements.

The clinical method was conducted by offering the subject a glass of drinking water prior to the trial procedure to quench their thirst as they were not be permitted any food, drink or smoke for 90 minutes after the photosensitizer mouth rinse application.
Chlorhexidine antiseptic mouthwash (conc. 2%) was given to the participant to cleanse the oral cavity of bacteria and plaque for 1-2 min prior to the ALA mouth rinse.
ALA mouth rinse was prepared by adding 50 ml of fresh or distilled water measured using hypodermic syringe to the contents of one Vial of ALA (1.5g). The Vial was shaken to ensure the ALA dissolved completely in the water, and then the content was divided into two equal amounts of (25ml) in plastic cups to be used for each patient. While the 1st patient used his mouth rinse, the other glass was kept in a dark place to ensure it was not affected by light.

The patients recruited would each get an ALA mouth rinse that they would have to rinse around their mouths for 15 minutes and after every 4-5 minutes they were allowed to spit out and refresh the rinse. They were encouraged to ensure the rinse wet all the mucosal surfaces. This procedure was repeated for 15 minutes and the time checked by using a digital timer clock.

The patient was asked to leave the clinic and to return after 90 minutes insisting that no food, drink or smoke could be consumed at this stage.

Before taking the measurements, the OBS was set and the references were stored by using dark piece of rubber blocked the end of the excitation and emission probe to subtract the background from the overall fluorescence spectra. Calibration performed by taking several readings of the backgrounds in the dark to establish a baseline reading

before each procedure. The background reading is automatically removed by the system from any future readings and true fluorescence was displayed. The graph lines and colours were displayed on the screen of the computer as shown in figures 4.1&5.

On return to the clinic the patient was sat on the chair and the room was made dark. The short wavelength Lamp (Wood's lamp and Torch lamp as shown in figures 4.6 & 4.7) were shone to see any glowing (fluorescence) of the lesion to be registered on the CRF for the comparison with results of the OBS probe.

The measurements were taken in the dark field, but the probe was guided intra orally with the aid of the short wavelength light (SWL) to detect the glowing areas and to identify the sites of the readings. In addition, to avoid any background light that may affect the measurements or potentially photo-bleach the photosensitizer.

The Torch Lamp was used to identify the boundaries of the oral lesions. For 19 patients, the lamp was used as an adjunct with the Woods lamp to assist "clinically" in detecting the glowing spots of the suspected sites and defining the lesion borders.

After the (SWL) readings were taken, Optical Biopsy System was used to take the measurements and the data saved on the memory of the laptop. Setting the OBS was done at the beginning of each patient to update the reference before taking the measurements. The next step was to store the fluorescence and set the lines on 500nm and 635nm (500nm was set by the soft wear as ALA autofluorescence and 635nm PpIX fluorescence) to be ready for the intra oral readings.

The readings were taken from

- Predetermined specific oral anatomical sites,
- The lesion and normal sites more than 5mm away from the lesion and recorded on a special form designed for this purpose (see appendix 6).

4.5 Readings Obtained for the Trial

When commencing the trial, the PI (Prof. Ogden) had taken the readings from the 1st three patients since the author had to be watching how to do the procedure and conduct the trial. The PI had observed difference in the intensity peaks of fluorescence between locations and when taking the readings from the normal tongue mucosa in particular. In view of this, it was thought necessary to obtain data from specific oral sites to investigate the influence of anatomical location on normal mucosal fluorescence.

4.5.1 Oral Anatomical Sites

To detect the variability of fluorescence intensity ratio (FIR), we investigated the influence of anatomical location on normal oral mucosal fluorescence characteristics to determine if site altered the spectral response.

Ten intra oral normal sites (anatomical sites) were chosen to obtain the readings to map out the oral cavity FIR and study the fluorescence tissue emission behaviour of the various oral mucosal types (keratinised and non keratinised mucosa).

The selected oral anatomical sites were clearly identified and represent the oral mucosal coating.

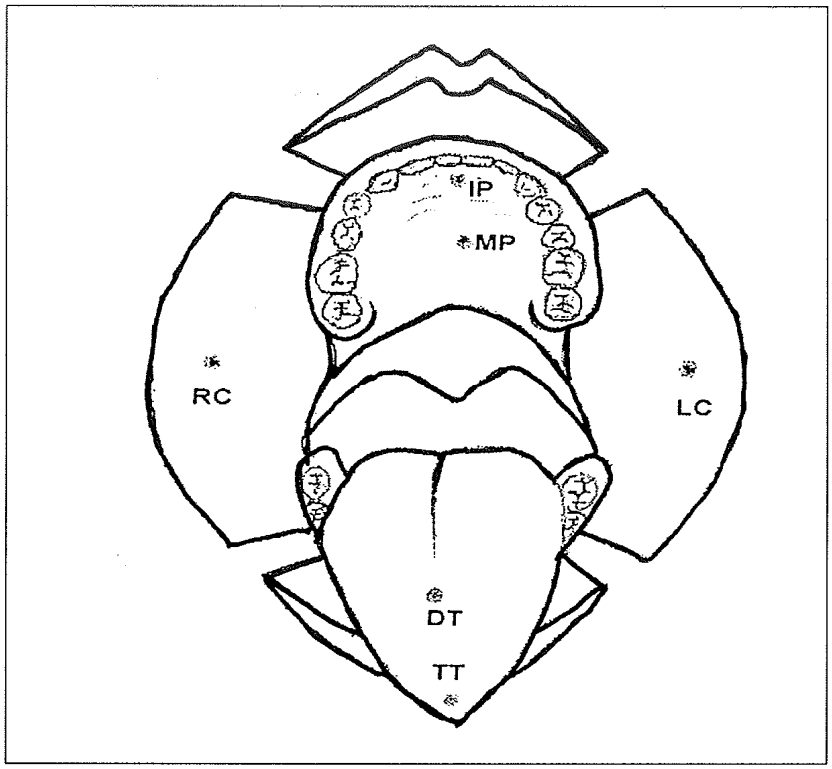
These points (as shown in Figure 4.9. A and B) were:

1. Incisive papilla palate.
2. Mid palate near the palatine suture.
3. 2mm away from the tip of the tongue.
4. 3cm on the dorsal surface of the tongue away from the tip.
5. 5mm from the ventral surface of the tongue away from the tip.
6. Floor of the mouth in the mid submandibular salivary gland orifices (Mid Wharton's duct).
7. Right cheek near the parotid opening.
8. Left cheek near the parotid opening.
9. Gingival site at the lower labial region above the lower lip fraenum attachment.
10. Lower lip above the fraenum attachment.

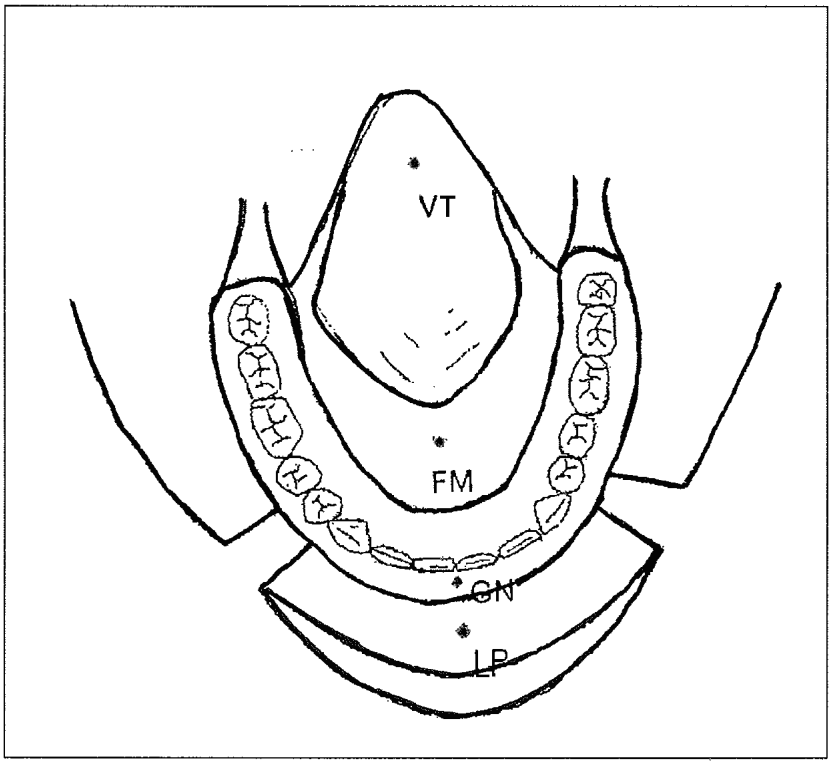
A list of these sites and the required biopsy sites readings were printed on a paper sheet and kept in the TMF records. The readings for every patient were kept with the CRF for archiving (see appendix 5).

The author has chosen the oral anatomical sites for the following reasons:

1- These sites included keratinized and non keratinized mucosa.
2- These mucosal surfaces are subjected to different mechanical, thermal (smoking) and microbial irritations.
3- Could be accessed precisely and repeatedly in obtaining the measurement from different patient.
4- Easy to access these sites by the probe in a darkened room.



A



B

Figure 4.9. A & B. Anatomical sites of the oral cavity. IP incisive papilla, MP mid palate, TT tip of tongue, DT dorsal tongue, VT ventral tongue, FM floor of mouth, RC right cheek, LT left cheek, GN gingival, LP lip.

4.5.2 Lesion Sites

The suggestion in the beginning of the trial was made to take five readings from each lesion. These readings were supposed to be taken from the upper, anterior, lower, posterior and middle sites of the lesion as designed in the protocol. The biopsy should excise the whole lesion so that all the reading sites could be included for the fluorescence and biopsy or histopathology comparison, however most of the surgical biopsy obtained from the lesions were 5mm in size (punch biopsy) that means it was not applicable in practice to include five readings in small size biopsy, for this reason it was rather more precise to take two readings from each 5mm biopsy site excised and examined under the microscope. However, all the readings obtained from a few excisional biopsies were included for the analysis and recorded as 1st and 2nd readings in the result tables.
Readings in a form of Excel files (saved and stored on the desktop of the computer) were taken from the biopsy site and one reading from the normal site.

The punch biopsy was taken from the most suspicious site clinically and with the highest fluorescence peaks of the OBS. The area from which to take the normal fluorescence readings "in theory" were the contralateral sites. However in practice a significant number of lesions were bilaterally distributed. Hence readings were taken from the same anatomical site of the oral cavity but more than 5mm away from the lesion borders.

Once the patient has finished the OBS readings, the biopsy was performed in the theatre room. The type of the surgical biopsy was decided depending upon the case; however punch biopsy was done for most of the patients with suspicious lesions.

The excisional biopsies were done in the theatre room under general anaesthesia, while the Punch biopsies were done in the clinic under local anaesthesia.

Punch biopsy was conducted by giving the patient local infiltration anaesthesia and biopsy was taken by using size 5mm punch biopsy knife. Vicryl silk suture was used to close the wound and instruction for wound healing was given to the patients. The patients were asked to come back two weeks later at Dundee Dental Hospital/Oral Medicine Clinic for review of any side effect of the drug, trial and surgical procedure.
The sample of tissue biopsy was fixed in 10% formalin and sent with the pathology information sheet to the pathology lab for the microscopic examination report.
The same procedure was followed in Glasgow.

The study was prospective in design and the fluorescence readings were collected based on the clinical and spectroscopic findings only. The definitive diagnosis used for the comparison was microscopic examination reported by the pathologist who had no pre report information about the spectroscopic findings.

4.6 Data Acquisition

The data obtained from the patients were Excel files for each reading representing the fluorescence intensity level of the assessed tissue site (lesion or normal).

The X axis of the graph (displayed on the screen of the computer and saved on the file) is representing the wavelengths of the spectra ranges from 339nm up to

1000nm (figure 4.5). The Y axis of the graph is representing the fluorescence intensity level of the tissue in each wavelength which is measured in arbitrary unit (a.u).

The spectra program of the instrument saves three fluorescence intensity readings at each wavelength so the average of each reading at 500nm and 635nm was taken and recorded in the tables (see appendix 7 table index 7.1).

The ratios were obtained by dividing the fluorescence intensity readings at 635nm and 500nm wavelengths (red/green) and the results displayed in the result's tables (see appendix 7 table index 7.2-7).

As mentioned before the system could provides us with the ratios of any wavelengths automatically by adjusting the curser over the column on the screen of the laptop using the laptop mouse pad manually at any wavelengths required for the assessment and displays this reading in a slot exists on the screen and on real time. However the ratios were taken from the Excel files for the following reasons;

- Working in the dark room hampered the accuracy in obtaining the results.
- Saving time writing the readings.
- Extension of the clinical or the chair work and increase the patient's suffering.
- Requires additional assistance at the time of the clinic.

4.7 Data Analysis

SigmaStat and SigmaPlot software packages were used for analysing the data and drawing the graphs for the results (Sigmaplot, 2010).

Descriptive analyses were conducted for median values of FIR at each site with graphical representation of the data. Box plot were graphed to observe the distribution of the values and the relations of the variables.

4.7.1 Anatomical Sites

Analysis of the ratios of the intensities at the red (635nm) and green (500nm) regions of the spectrum was made (table index 3). An analysis of variance was performed to determine whether the average peak fluorescence intensity ratios for all normal oral mucosa differed among sites.

The data were analysed using one way ANOVA to compare the relation of the fluorescence intensity ratios of the readings obtained from the patients as a test variable and the anatomical sites as a factor.

4.7.2 Individual Characteristics

The same set of spectral data obtained from each patient were analyzed to detect variance in the fluorescence intensity ratio at the sites (categorised in relations to the anatomical sites) and the individual characteristics which include the age, gender,

and presence of the metabolic disease, presence of dental prosthesis, smoking and alcohol consumption (see appendix 7 table index 7.4&5).

A t test was used to compare the relation between each character that was normally distributed and Mann-Whitney test used to test a non-normally distributed data.

- The age groups were classified into young (less than 50 years) and old (above 50 years) to compare the FIR between the age groups.
- The gender was grouped into male and female groups.
- The systemic diseases considered in the comparison were metabolic diseases that might influence the fluorescence of the tissues and the diseases included were diabetes and thyroid (hypo or hyper) function.
- Presence of removable or fixed prosthesis and absence of prosthesis were considered in the comparison of the FIR between the two groups analysis.
- The effect of smoking: smokers (smoke at least one cigarette a day) were compared to non smokers.
- Alcohol was defined as any regular intake (regardless of the number of the units) compared to those who abstained (this was because of the difficulty of reliability assessed exact alcohol intake)

4.7.3 Lesions and Normal Sites

Fluorescence intensity ratios at 635/500nm (red/green) from abnormal sites were compared with those from normal sites.

Readings of the spectra were obtained from the lesions were categorised into 1st and 2nd readings (see appendix 7 table index 7.6&7.7), however in table index 6.7 more than two readings were taken from the excised lesions, they were listed in two categories and considered as lesion site readings.

One way ANOVA was applied to compare the two readings from the lesion and normal sites (table index 7). Further analysis was done by merging the two readings of the lesion to be considered as a disease sites and compared with the normal sites by using Mann-Whitney test (non normal distribution test) to detect the difference between the lesion and normal sites.

Readings obtained from the normal sites were used for the comparison and the lesions were classified in a range of lesions based on the histopathological diagnosis. The diagnosed lesions were grouped into three categories for the results analysis:

- Benign hyperkeratosis; were lesions histologically showed no evidence of inflammatory reaction.
- Inflammatory keratotic lesions; were red and white lesions showed inflammatory cell infiltration such as lichen planus or lichenoid lesions and/or superimposed by fungal infections.
- Dysplastic; were lesions showed evidence of dysplasia without considering the stage and neoplasm.

Spectra line graphs were averaged in relation to the sample size and displayed in the result graphs to assist in spectra comparison interpretation.

Sensitivity and specificity as well as the predictive values of the Fluorescence Intensity Ratio (FIR) in the detection of the premalignant lesions were also assessed. e detection of the premalignant lesions were also assessed.

Chapter 5

Results

A total of 35 patients were recruited and consented. They were 27 patients recruited at the Dundee site (Ninewells hospital/Clinical Research Centre) and 8 patients at the Glasgow site (Southern Glasgow hospital).

Limitation in recruiting patients and problems associated with conducting a CTIMP will be discussed later.

A total of 426 spectra were obtained from 35 patients recruited for the trial (see appendix 7 index tables). The spectra were light emitted and collected by the compact fluorescence spectroscopy from the tissue site and stored by the computer in a form of Excel files. Each Excel file contains spectra readings from one tissue site saved and stored in the computer of the instrument.

The level of 500nm (green) peak indicates the autofluorescence tissue emission which is expected to be high because it represents fluorescence emission reflected from the normal tissue. While 635nm (red) peak indicates PpIX fluorescence that (as explained previously is associated in theory with a change toward malignant phenotype) occurs due to alteration in the chemical or structural components of the tissues. High fluorescence intensity ratio (635/500nm or red/green ratio) indicates alteration in the normal tissue fluorophore emission due to PpIX accumulation resulting from the cellular metabolism of 5-ALA (assumed not to occur in normal, non diseased tissue).

Management of the FIR was done by comparing all the readings as raw data without exclusion of the extreme values (outlier), while very extreme values were excluded in drawing the graphs only using SigmaStat or SigmaPlot package.

5.1 Fluorescence intensity ratio (FIR) measurement at the oral anatomical sites

A decision to map out the fluorescence intensity ratios of the normal oral mucosal sites was made after noticing false positive readings from some areas, of the normal oral mucosa and of the tongue in particular, for the 1st three patients recruited for the trial. For this reason, these patient's readings were excluded from the trial analysis since their readings were not taken from the exact sites that were chosen for the patient in the rest of the study.

As a result of these observations, a total of 292 spectra (readings) were taken from 10 oral anatomical sites (figure 4.9) of 32 patients consented and recruited for the trial (see appendix 7 table Index 7.1, 2&3).

The gender of the patients was 16 males and 16 females. The age ranged from 21-84 with the mean age 59.2 years and Standard deviation 15.3 years.

Ten sites were selected to compare the FIRs and the sites included for this study were examined clinically and spectroscopically. The oral anatomical sites were listed in a sheet (see appendix) designed to record the observations and stored in the trial master file (TMF) and the data were saved in the computer (University web in compliance with the SOP) for the results analysis.

Table Index 7.1 (see appendix 7) showed the fluorescence readings of each patient at 500nm and 635nm at 10 anatomical sites.

Table Index 7.2&3 showed the fluorescence intensity ratios at the anatomical sites for each patient. The ratios of the readings were obtained by dividing the value of the fluorescence intensity at 635/500 which represents the red/green ratio and the values of each ratio were recorded. This value could be obtained from the computer screen of the instrument or from the excel data file. There were data taken but not included (IN) and missing or not obtained (NO) values, since the sites were not fit the criteria of site selection or due to technical errors.

Descriptive statistics were calculated for each variable. The variables in the study were fluorescence intensity ratio at 635/500nm (red/green ratio) of the normal mucosa at different anatomical sites (table 5.1).

Anatomical Site	No. of Patients	Minimum	Maximum	25%	75%	Median	Mean	Standard Deviation
Incisive Papilla (IP)	32	0.440	5.570	0.855	2.675	1.760	1.948	1.295
Mid Palate(MP)	26	0.1000	4.120	0.670	2.160	1.130	1.439	1.049
Tip Tongue 2mm (TT)	31	0.350	3.940	0.910	2.598	1.480	1.703	0.987
Dorsal Tongue 3cm (DT)	32	0.360	4.840	0.815	1.695	1.225	1.519	1.060
Ventral Tongue (VT)	25	0.210	5.610	0.410	1.093	0.580	1.043	1.218
Floor Mouth (FOM)	31	0.140	3.380	0.397	1.475	0.610	1.028	0.911
Right Cheek (RC)	32	0.120	1.730	0.250	0.900	0.470	0.613	0.433
Left Cheek (LC)	31	0.1000	4.900	0.285	1.210	0.710	0.868	0.943
Gingiva (GN)	26	0.150	5.140	0.530	1.280	0.865	1.112	1.011
Lip	26	0.220	3.090	0.630	1.710	0.865	1.179	0.831

Table 5.1. Summary of the data statistical description. Sample size ranged between 26 and 32 patients.

An overview of the findings was made. Median values were noticeably lower than mean in most measurements which indicates that the data were skewed and non-normal in distribution. The inter-quartile range of the 25% and 75% were recorded to show the range of the distribution of the data which varied between sites, as a result, the median values of the FIR are more reliable since they are less affected by the extreme values.

Highest and lowest median values ranged between (1.76 – 0.47) with the highest value that of the IP followed by TT, DT, MP, GN, LIP, LC, FOM, VT and the lowest was of the RC respectively.

The mean values of the normal anatomical sites did not vary that much with the median, maximum and minimum values. The highest median and mean FIR values of the IP were 1.76 and 1.948 followed by the tongue (1.480, 1.703) and the lowest were that of the RC (0.47, 0.613). There was a variance in the dispersion in the values which can be demonstrated by the differences in the standard deviation of the different sites which indicates the differences in the FIR at some sites.

SigmaStat or SigmaPlot package was used to obtain the values (*p* values) which stands for the probability of difference.

The significance of the difference in the FIR was obtained by applying Analysis of Variance (ANOVA) which recommended Holms sidak method as a powerful procedure used for both pairwise and multiple comparison testing and to determine which pairs of sites tend to be different e.g. IP vs. RC. The outcome shows the differences in the mean values among all the groups were greater than would be expected by chance; there was a statistically significant difference ($P = <0.001$) between all the groups and summery of the results is shown in table 5.2.

Source of Variation	DF	SS	MS	F	P
Between Groups	9	45.922	5.102	5.151	<0.001
Residual	282	279.332	0.991		
Total	291	325.254			

Table 5.2. Summary of the result analysis among the groups. Degree of Freedom DF, Sum square SS, Mean square MS, F and *p* values.

There were some sites that did not differ from each other in comparison between all the groups while others shown highly significant difference (P =0 .001). To explore more in depth the difference of the FIR at each anatomical site, the following tables illustrate the variability of FIR at the oral anatomical sites using two levels of significance (0.05 and 0.001). The pairwise comparisons between the anatomical site means, t values, unadjusted *p* value and level of significance at 0.05 and 0.001 level of each anatomical site measured are shown in the following tables (Table 5.3, A-I).

Table 5.3 (A-I). Summary of the oral anatomical site fluorescence intensity ratio using 0.05 and 0.001 statistical levels.

A-Incisive Papilla (IP)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**IP vs. RC**	1.335	5.365	0.000000169	Yes	Yes
2	**IP vs. LC**	1.080	4.306	0.0000229	Yes	Yes
3	**IP vs. FOM**	0.920	3.667	0.000293	Yes	Yes
4	**IP vs. VT**	0.905	3.405	0.000757	Yes	Yes
5	**IP vs. GN**	0.836	3.180	0.00164	Yes	No
6	**IP vs. LIP**	0.769	2.926	0.00371	Yes	No
7	**IP vs. MP**	0.509	1.935	0.0539	No	No
8	**IP vs. DT**	0.429	1.723	0.0860	No	No
9	**IP vs. TT**	0.245	0.978	0.329	No	No

Table 5.3. A. shows that IP has FIR levels higher than most of the oral anatomical sites and this is clear when compared at both 0.05 and 0.001 level of significance. There is a significant difference at 0.05 with RC, LC, FOM, VT, GN, and LIP; however there is no significant difference with MP, TT and DT. There was no significant difference between the three sites and the IP (at P=0.001) which had similar FIRs high when compared with the rest of the oral sites.

B-Tip of the Tongue (TT)

No	Comparison	Diff of Means	T	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**TT vs. RC**	1.090	4.345	0.0000195	Yes	Yes
2	**TT v s. LC**	0.835	3.302	0.00108	Yes	Yes
3	**TT vs. FOM**	0.675	2.668	0.00807	Yes	No
4	**TT vs. VT**	0.659	2.465	0.0143	Yes	No
5	**TT vs. GN**	0.590	2.230	0.0265	Yes	No
6	**TT vs. LIP**	0.524	1.979	0.0488	Yes	No
7	**TT vs. MP**	0.263	0.995	0.321	No	No
8	**TT vs. DT**	0.184	0.732	0.465	No	No

The FIR of TT was high when compared with other sites at level 0.05. This was particularly so when compared with the RC and LC. The least was with the lip. The difference of the FIR of the TT was non significant if compared with the MP and DT.

C-Dorsal Tongue (DT)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**DT vs. RC**	0.906	3.642	0.000322	Yes	Yes
2	**DT vs. LC**	0.651	2.597	0.00990	Yes	No
3	**DT vs. FOM**	0.491	1.958	0.0513	No	No
4	**DT vs. VT**	0.476	1.791	0.0743	No	No
5	**DT vs. GN**	0.407	1.548	0.123	No	No
6	**DT vs. LIP**	0.340	1.295	0.196	No	No
7	**DTvs. MP**	0.0798	0.304	0.762	No	No

The FIR readings of DT was high when compared with RC and LC, however there was no significant difference at both 0.05 and 0.001 when compared with the other oral sites.

D-Mid Palate (MP)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**MP vs. RC**	0.826	3.145	0.00184	Yes	No
2	**MP vs. LC**	0.571	2.159	0.0317	Yes	No
3	**MP vs. FOM**	0.411	1.554	0.121	No	No
4	**MP vs. VT**	0.396	1.421	0.157	No	No
5	**MP vs. GN**	0.327	1.184	0.237	No	No
6	**MP vs. LIP**	0.260	0.943	0.346	No	No

MP readings were significantly different at 0.05 when compared with RC and LC, however the FIR was not significant different from other sites of the oral cavity at 0.001 level.

E-Lip

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**LIP vs. RC**	0.566	2.154	0.0321	Yes	No
2	**LIP vs. LC**	0.311	1.175	0.241	No	No
3	**LIP vs. FOM**	0.151	0.570	0.569	No	No
4	**LIP vs. VT**	0.136	0.487	0.627	No	No
5	**LIP vs. GN**	0.0665	0.241	0.810	No	No

The FIR of LIP was the least highly ranked when compared with other oral sites. There is only significant difference at 0.05 when compared with RC.

The rest of the oral sites GN, VT, FOM, LC and RC had no significant difference in the FIR when compared with one another in a pairwise at both levels of significance.

F-Gingiva (GN)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**GN vs. RC**	0.499	1.901	0.0583	No	No
2	**GN vs. LC**	0.245	0.924	0.356	No	No
3	**GN vs. FOM**	0.0842	0.318	0.750	No	No
4	**GN vs. VT**	0.0691	0.248	0.804	No	No

G-Ventral Tongue (VT)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**VT vs. RC**	0.430	1.620	0.106	No	No
2	**VT vs. LC**	0.175	0.656	0.512	No	No
3	**VT vs. FOM**	0.0151	0.0566	0.955	No	No

H-Floor of the Mouth (FOM)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**FOM vs. RC**	0.415	1.656	0.0989	No	No
2	**FOM vs. LC**	0.160	0.634	0.526	No	No

I-Left Cheek (LC)

No	Comparison	Diff of Means	t	Unadjusted P	Level 0.05 Sig	Level 0.001 Sig
1	**LC vs. RC**	0.255	1.016	0.310	No	No

Figure 5.1 shows the distribution of the data at different anatomical sites. The variation was expressed by the inter quartile range between the different locations and the median values between IP, TT, DT in particular, which were spectrally distinct and to some extent with MP and LIP relatively different, while the other spectral line graphs were overlapped and the values showed no statistically significant difference between the groups.

In viewing the graphs, relevant systematic spectral differences was observed between IP, DT and TT, while not for the rest of the oral sites (VT, FOM, RC, LC, GN, Lip i.e. the non keratinized sites).

Spectra interpretation

The technique of the OBS and the outcome graph results (figure 4.5) was discussed in the Patients, Material and Method section. The fluorescence line graph started (the recorded data) from 339nm up to 1020nm wavelength (pre-programmed OBS system). The spectrometer had a wavelength range of 430 to 730nm and the acquired data were analysed by the computer to display the final graph.

Figure 5.3 shows the averaged spectra obtained from the anatomical sites of all the participants. The line graphs were displayed in colours to discriminate the spectra excitation and emission fluorescence intensity level of each anatomical site.

The line graphs started at 405nm wavelength (excitation wavelength of the diode laser emitted by the OBS) and the outcome did not show data till 430nm where the line graphs were noticed start rising up due to cellular emission of light in a blue wavelength range which was supposedly induced by certain endogenous fluorophores (mentioned in the review of lit figure 1.3.8 and 1.3.9). The endogenous fluorophore emissions (autofluorescence) continue rising and reach a peak at 500nm (green wavelength). The line graphs at this level showed a variation in the autofluorescence intensity level between the different oral locations with the highest to the lowest being RC, LC, GN, FOM, LIP, DT, TT, IP, VT and MP respectively. The fluorescence intensity level for all the anatomical sites then drops down when the wavelength reaches 600nm since there is no autofluorescence emission. At 635nm the fluorescence intensity peaks rises again up to the peak which assumed to represent the PpIX emission wavelength. The emission ranged (from highest to lowest) IP, TT, DT followed by GN, LIP, MP, FOM, VT, LC, and RC respectively.

There were overlaps in both autofluorescence and PpIX fluorescence emission peaks in some anatomical locations as shown in the graphs of figure 5.3.

Normalisation of the spectra by transforming the data to the logarithm value is reliable method for statistical analysis and graphic interpretation. Graphs after normalisation of the spectra are shown in figure 5.4.

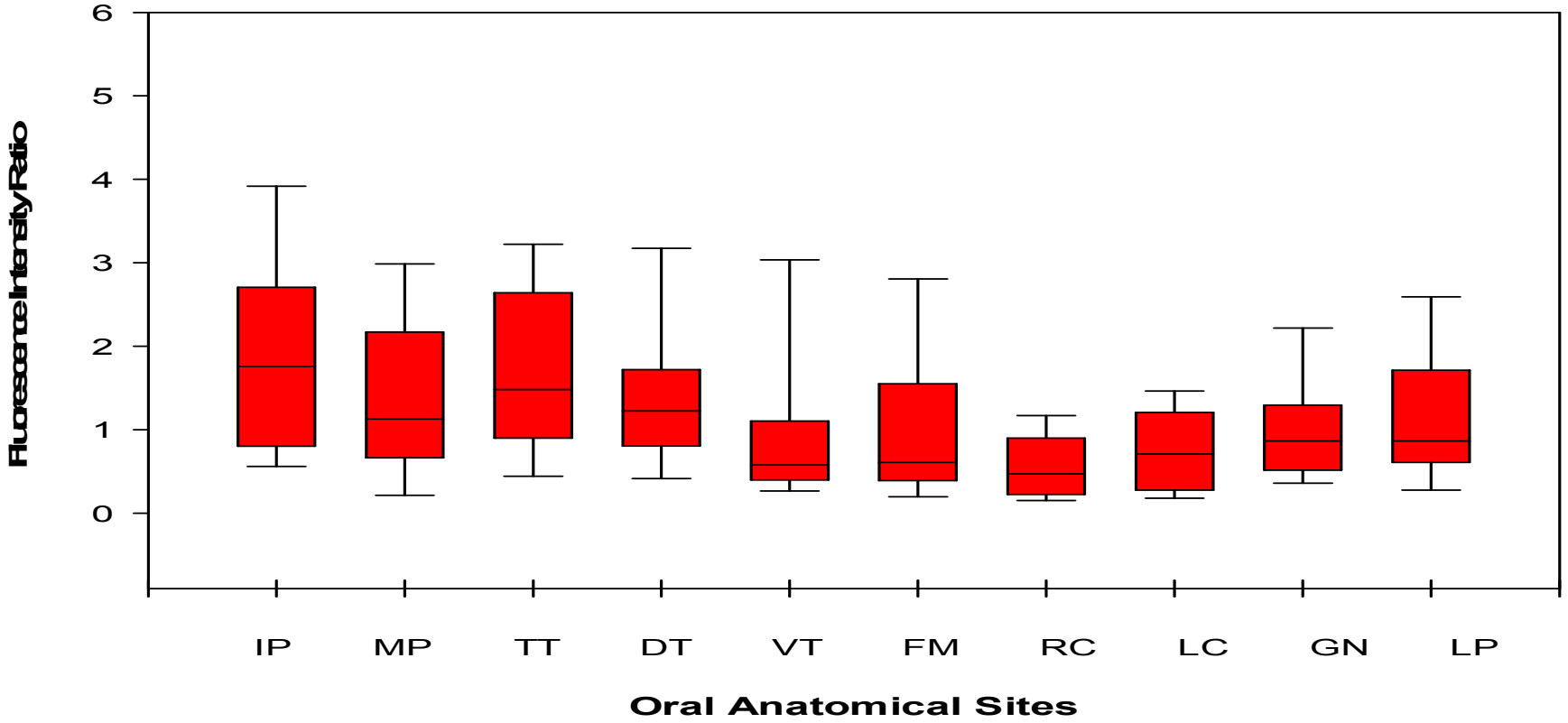


Figure 5.1. Fluorescence intensity ratio at (635/500nm) or red/green ratio of the normal oral mucosa at different anatomical sites. IP incisive papilla, MP mid palate, TT tip of the tongue, DT dorsal tongue, VT ventral tongue, FM floor of the mouth, RC right cheek, LC left cheek, GN gingiva, LP lip.

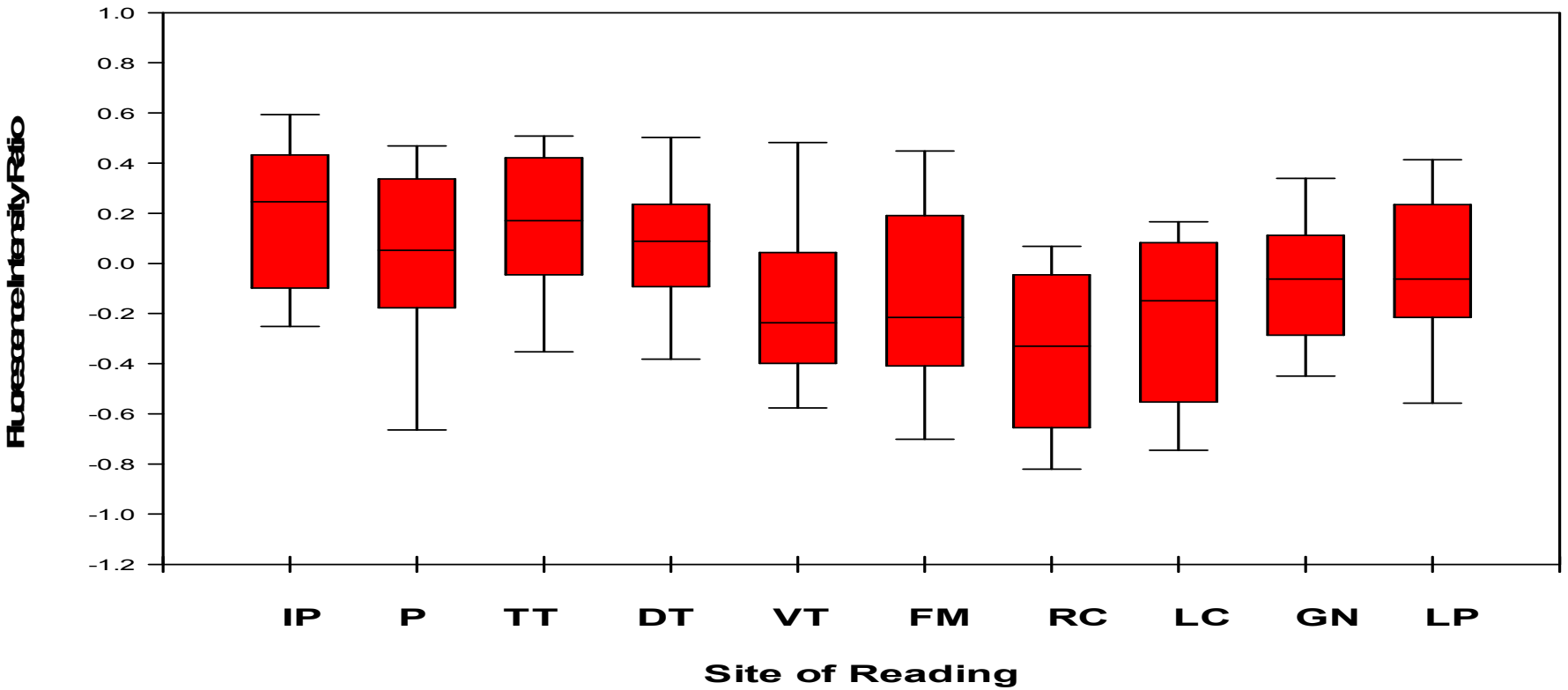


Figure 5.2. Normalization of fluorescence intensity ratio data of the normal oral mucosa at different anatomical sites. IP incisive papilla, MP mid palate, TT tip of the tongue, DT dorsal tongue, VT ventral tongue, FM floor of the mouth, RCright cheek, LC left cheek, GN gingiva, LP Lip.

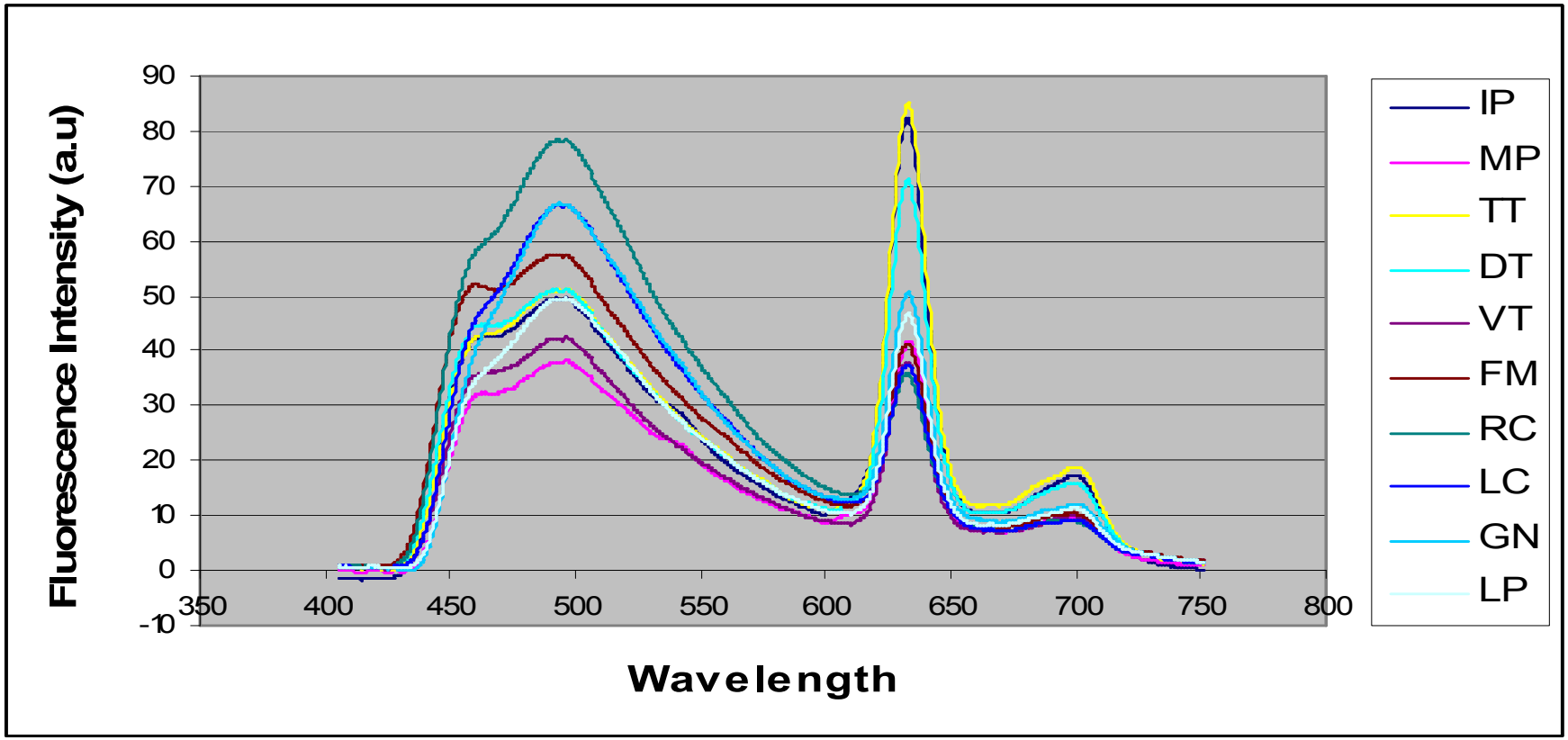


Figure 5.3 Average fluorescence spectra excitation at (405) and emission recorded from the oral anatomical sites. IP incisive papilla, MP mid palate, TT tip of the tongue, DT dorsal tongue, VT ventral tongue, FM floor of the mouth, RC right cheek, LC left cheek, GN gingiva, LP Lip.

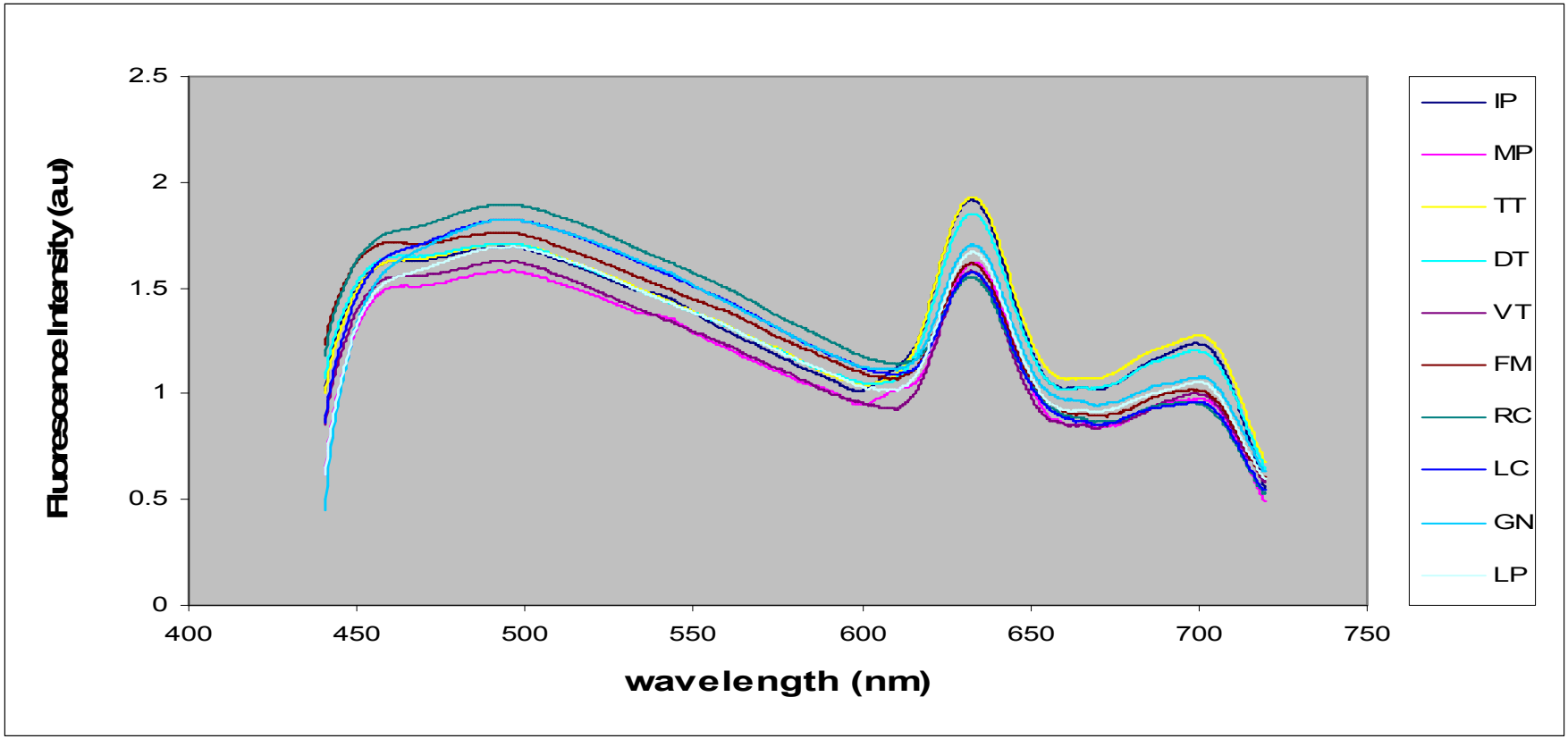


Figure 5.4. Average-scale, normalized fluorescence spectra recorded from the oral anatomical sites. IP incisive papilla, MP mid palate, TT tip of the tongue, DT dorsal tongue, VT ventral tongue, FM floor of the mouth, RC right cheek, LC left cheek, GN gingiva, LP Lip.

5.2 Fluorescence intensity ratio (FIR) measurements in relation to the individual characteristics

As stated before, we have observed variations in person-to-person fluorescence intensity peaks at the same oral anatomical sites. This observation suggested that tissue fluorescence is altered due to variation in individual characteristics.

Our hypothesis is that the alteration in the FIR was corresponded to the variation in individual characteristics such as;

- Age
- Gender
- Presence of dental prosthesis
- Presence of metabolic disease
- Smoking habit
- Alcohol consumption

The same set of data obtained from the oral anatomical sites of 32 patients which were 292 spectra readings were utilized for this part of result analysis (see appendix 7 table Index 7.4).

The age ranged 21-84 with the mean age 59.2 years and Standard deviation 15.3 years. The information regarding age, sex, medical and dental history and habits were obtained from the patients and recorded in the CRFs (case report forms), in addition to a special form designed by the author for this purpose (see appendix 4&5), finally tabulated and used for the statistical analysis.

Unfortunately, the Glasgow site didn't comply with the instructions to collect some of this information, so that the non-obtained data were treated as missing values.

Table Index 7.4 showed the individual characteristics and the fluorescence intensities at 500nm and 635nm of the oral anatomical sites for each patient.

The 1st three patients' readings (PDD01, 02, 03) were not included because they were not taken from the sites chosen to be tested, therefore indicated as NI. The non obtained values were indicated as NO in the previous tables, however in these tables were indicated as missing Mi to avoid confusion with the non-parametric values of the individual characteristics (Yes & No and M&F).

Table Index 7.5 showed the individual characteristics and the approximated FIR values (for statistical analysis) of the oral anatomical sites for each patient.

5.2.1 Comparison of FIR measurements of the oral anatomical sites between the two age groups

A total of 292 FIR measurements obtained from the anatomical sites of 32 patients were utilized for this part of study.

Since the number of the patients was limited, and of the young age group in particular, we have classified the ages into two groups, less than 50 years and over 50 years and for the comparison.

Table 5.4 showed the statistical description of the FIR readings of both age groups. It was observed that there was variation in the mean and median values between the age groups. The inter quartile range and the standard deviation of the data for both

groups were similar which indicate the FIR readings were distributed approximately in the same pattern.

Age groups	No. of Patients	No. of FIR read	Max	Min	Mean	Median	25%	75%	Std Dev
Less than 50	9	86	4.240	0.1000	1.341	1.070	0.480	1.730	1.085
over 50	23	206	5.610	0.1000	1.213	0.900	0.470	1.500	1.046

Table 5.4. Descriptive statistics summary of the FIR measurements of the oral anatomical sites obtained from both age groups.

Comparison of the FIR readings between the two groups (male and female) using Mann-Whitney Rank Sum Test (non normal distribution data) found the difference in the median values between the two groups was not significant (P = 0.446). Therefore, there was not a statistically significant difference ($p>0.05$) between the FIR of the oral anatomical site readings obtained from the two age groups.

Comparison of the FIR values distribution; inter quartile range and the median values of the ages less than 50 and over 50 were shown in figure 5.5.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the age group. They were classified into two age groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison as shown in figure 5.6.

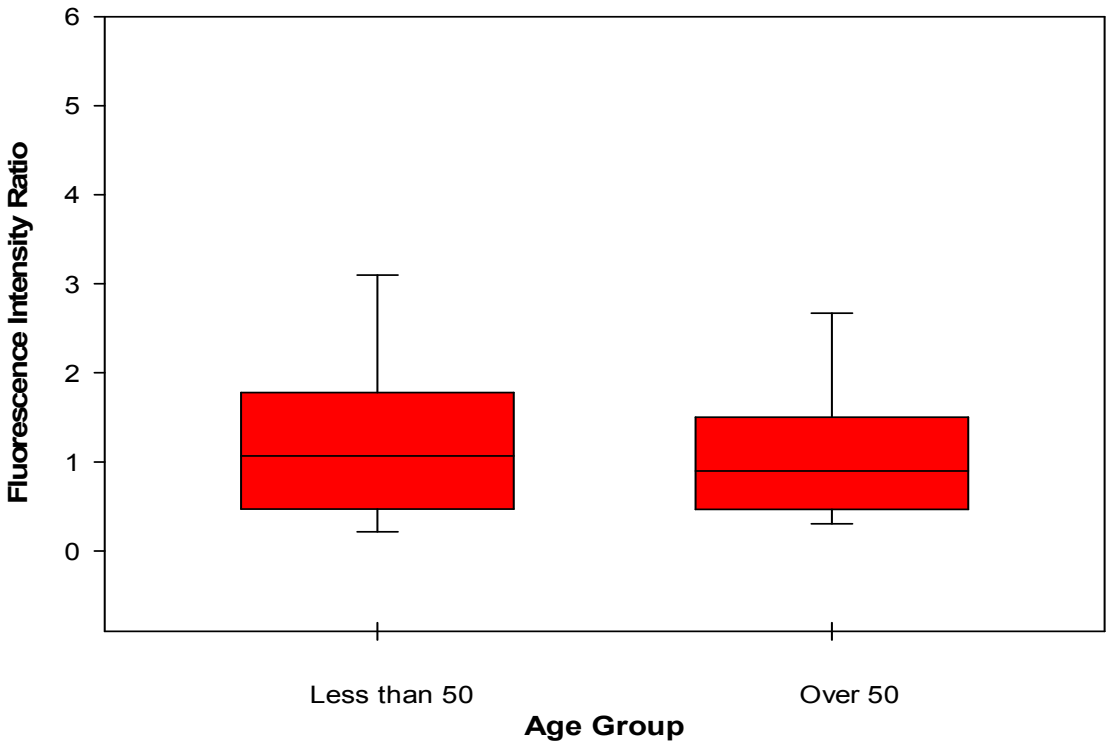


Figure 5.5. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to age groups.

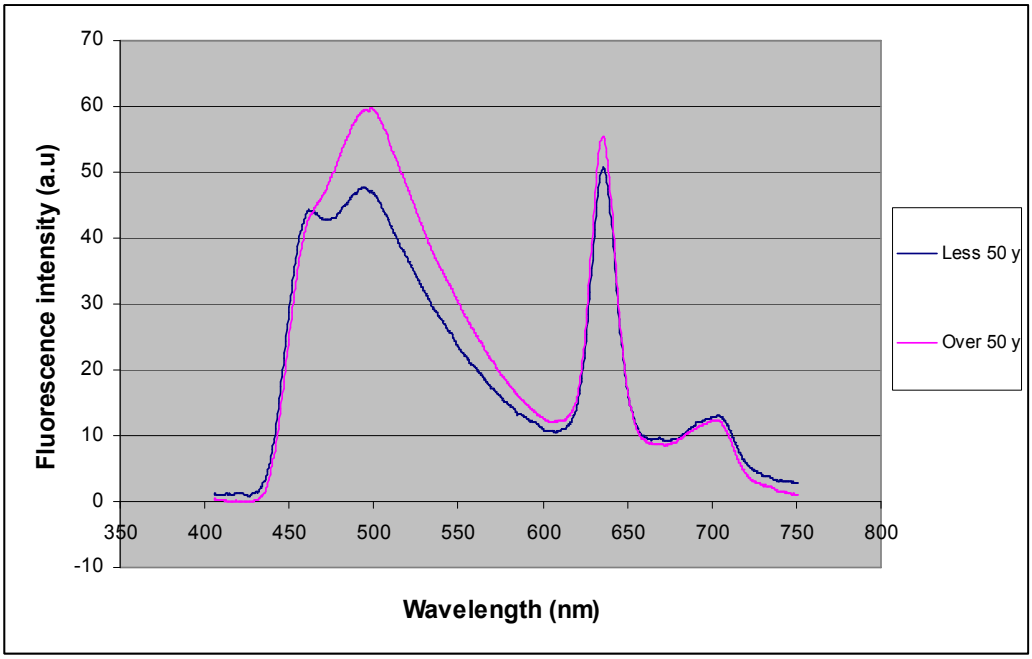


Figure 5.6. Average fluorescence spectra of all oral anatomical sites of the two age groups.

5.2.2 Comparison of FIR measurements of the oral anatomical sites between the two genders

Gender of the patients included for this part of the trial was 16 males and 16 females. The FIR measurements of the data sets were divided into two groups according to the sex of the patient. The data for this analysis were obtained from table Index 7.5.

The FIR measurements obtained from all the anatomical sites of the males were compared with that of the females.

A total of 292 readings (FIR) were analysed statistically obtained from 32 patients. Table 5.5 showed the statistical description of the FIR readings of both genders. It was observed that there was a very slight difference in the mean and median values between male and female. The inter quartile range and the standard deviation of the data for both genders were approximately have the same pattern, which indicates closely similar distribution of the FIR readings (figure 5.7.).

Gender	No. of Patients	No. of FIR read	Max	Min	Mean	Median	25%	75%	Std Dev
Male	16	152	5.610	0.1000	1.254	0.850	0.470	1.650	1.136
Female	16	140	5.140	0.180	1.244	0.950	0.460	1.590	0.971

Table 5.5. Descriptive statistics summary of the FIR measurements of the oral anatomical sites obtained from both genders.

Comparison of the FIR readings between the two groups (male and female) using Mann-Whitney Rank Sum Test (non normal distribution data) found the value (P = 0.455) was no statistically significant difference (p>0.05) between the two genders.

Comparison of the FIR values distribution; inter quartile range and the median values of the male and female are shown in figure 5.7.

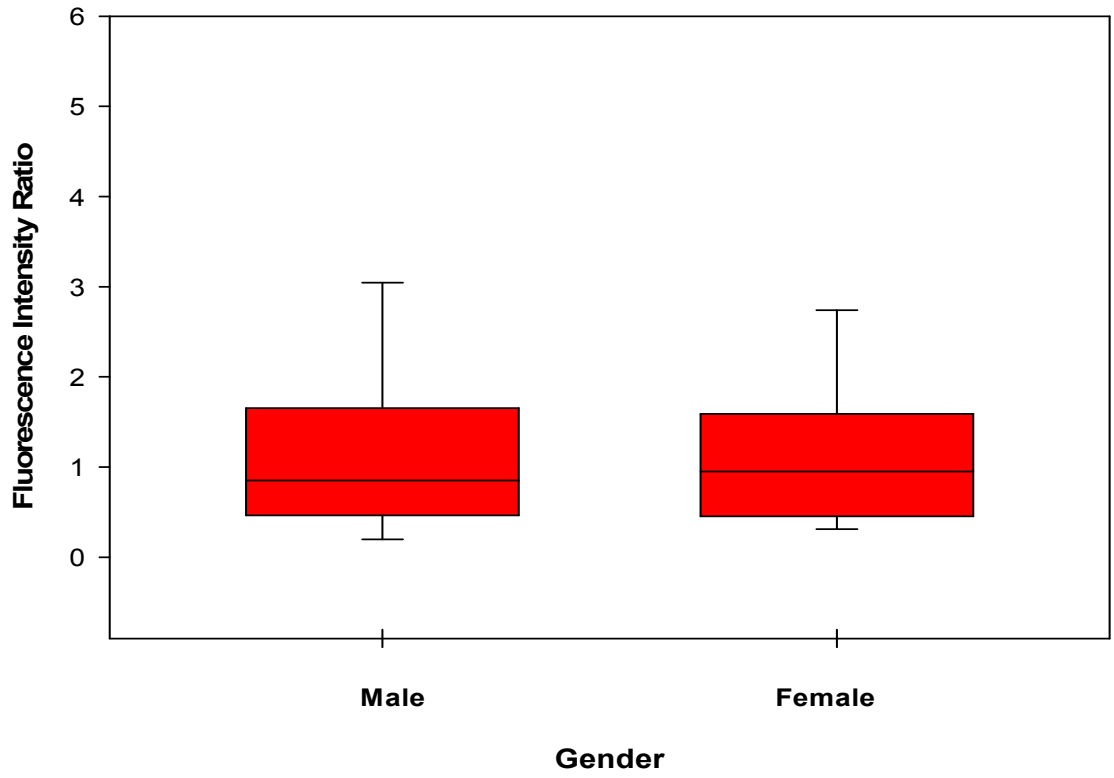


Figure 5.7. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to gender.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the gender. They were classified into male and female groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison. It has been noticed there is difference in the line graphs between

male and female as far as the autofuorescence and PpIX fluorescence, however the peak fluorescence intensity ratios were relatively approximate in relation to the values as shown in figure 5.8.

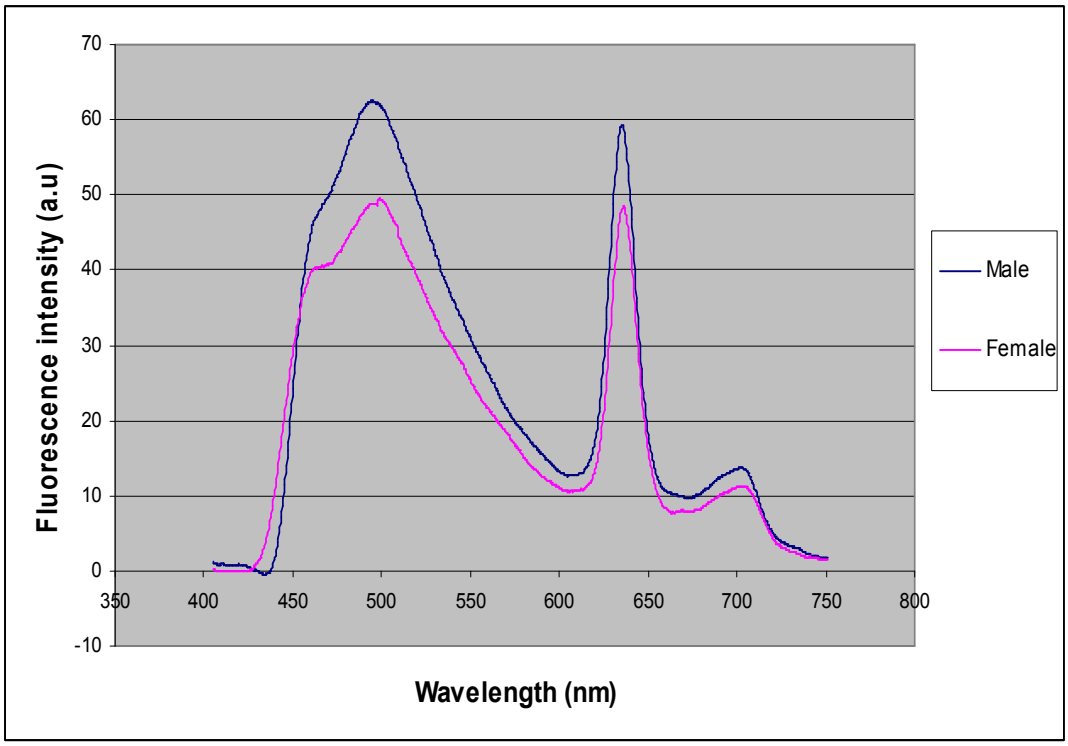


Figure 5.8. Average fluorescence spectra of all oral anatomical sites of the two genders.

5.2.3 Comparison of FIR measurements of the oral anatomical sites in relation to the presence of dental prosthesis

Presence of removable or fixed prosthesis was anticipated as a factor might have influence on the oral mucosal fluorescence due to microbial accumulation, physical irritation and the inflammatory consequences of the tissues.

The FIR measurements obtained from all the anatomical sites of the cases with dental prosthesis were compared with that of the non prosthesis.

A total of 218 readings (FIR) obtained from 24 patients were analysed statistically. The patients who were using prosthesis and included for the analysis were 12 and patient without prosthesis were12 too.

Presence of one or both removable prosthesis and any fixed prosthesis regardless of the number of units was considered as present (prosthesis), otherwise considered absent (non prosthesis). The FIR readings statistical description of the patients with prosthesis and non prosthesis are listed in table 5.6.

State	No of Patients	No. of FIR read	Max	Min	Mean	Median	25%	75%	Std Dev
Prosthesis	12	108	5.570	0.120	1.145	0.900	0.465	1.480	0.939
Non Prosthesis	12	110	5.610	0.1000	1.512	1.125	0.540	2.270	1.269

Table 5.6. Descriptive statistics summary of the FIR measurements of the oral anatomical sites of patients with prosthesis and non prosthesis.

Comparison of the FIR between the patients with prosthesis and non prosthesis, using Mann-Whitney Rank Sum Test, found that the value (P = 0.063) was no statistically significant difference (p>0.05) in the FIR readings between the two groups. Comparison of the FIR values distribution, inter quartile range and the median values of the prosthesis and non prosthesis are shown in figure 5.9.

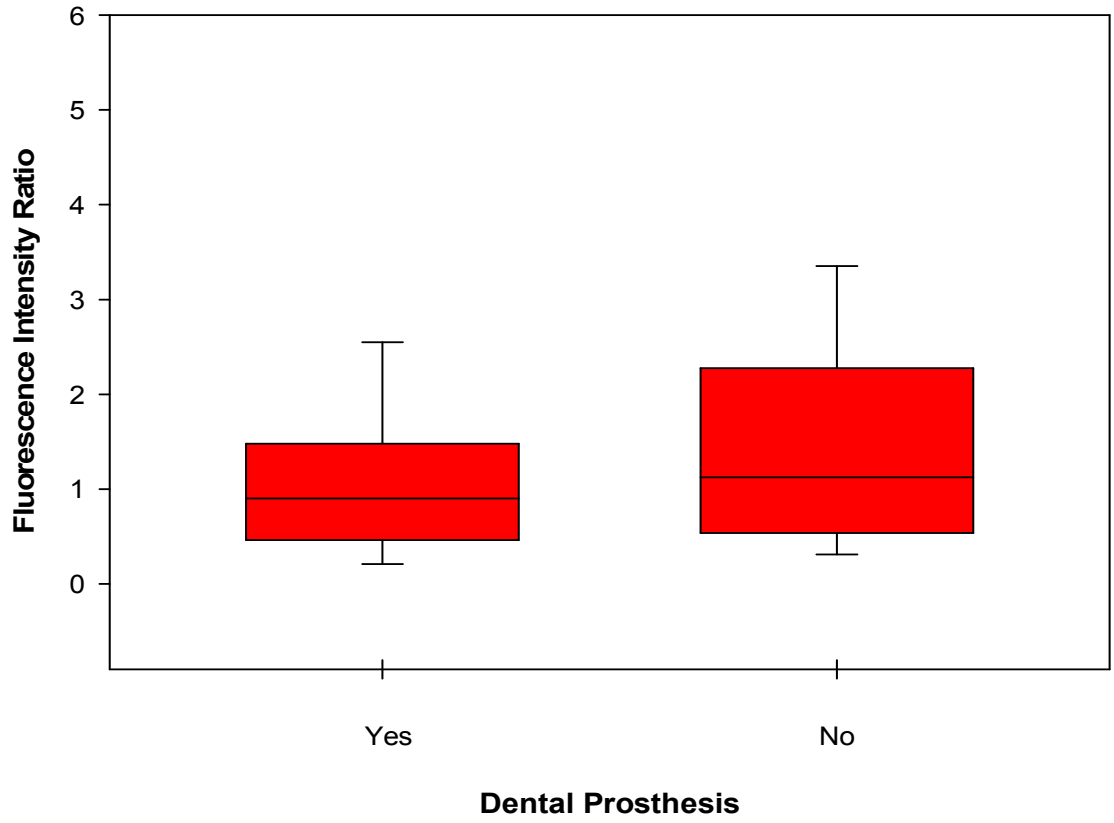


Figure 5.9. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to presence of dental prosthesis.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the presence or absence of dental removable or fixed prosthesis. They were classified into prosthesis and non prosthesis groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison. The line graphs show noticeable variation in the PpIX fluorescence which indicates the lower red green ratio of the presence of prosthesis spectrally as shown in figure 5.10.

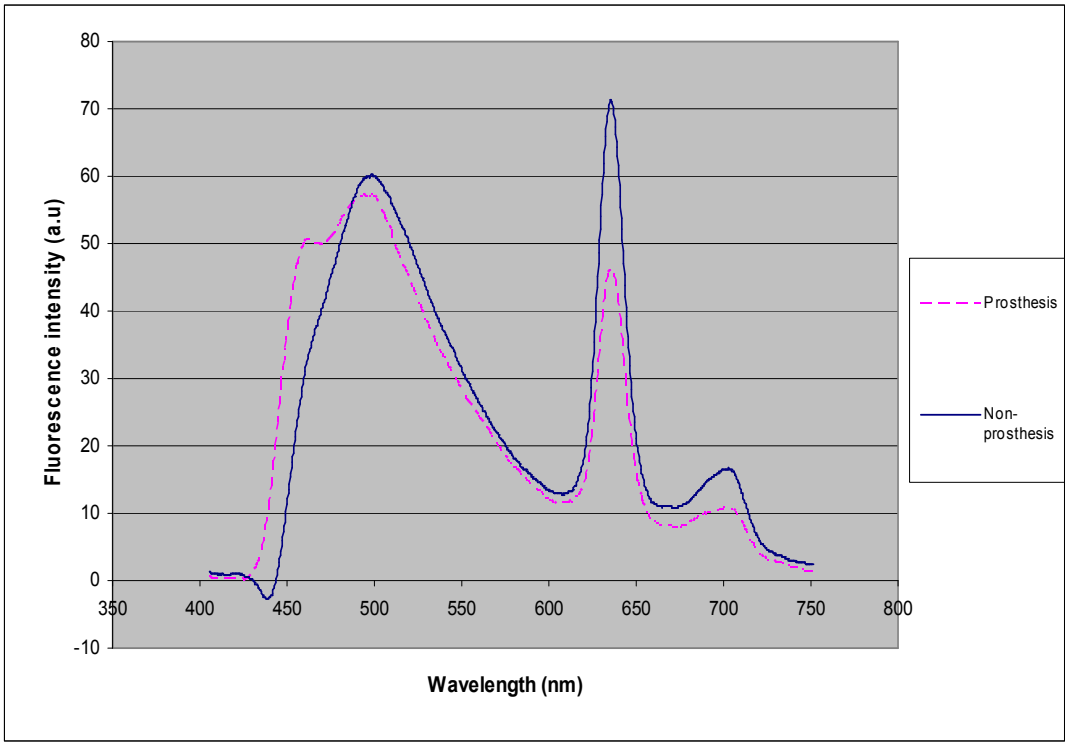


Figure 5.10. Average fluorescence spectra of all oral anatomical sites in relation to presence of dental prosthesis.

5.2.4 Comparison of FIR measurements of the oral anatomical sites in relation to presence of the metabolic diseases

History of the systemic diseases was collected from the patient recruited for the trial. Metabolic diseases like diabetes and thyroid (hypo or hyper) function were suspected to have influence on the cell chemical or structural components.

The FIR measurements obtained from all the anatomical sites of the cases who were having metabolic diseases was compared with that who had metabolic diseases was compared with those who did not have metabolic disease.

A total of 218 readings (FIR) were analysed statistically, obtained from 24 patients. They were 192 readings obtained from 20 recruited cases that didn't have

metabolic diseases and 26 readings obtained from 4 having metabolic diseases. The cases that had diseases were indicated as disease, while the others as non disease in the summary table (table 5.7).

Status	No. of Patients	No. of FIR Readings	Max	Min	Mean	Median	25%	75%	Std Dev
Disease	4	26	2.740	0.320	1.062	0.875	0.510	1.220	0.716
Non Disease	20	192	5.610	0.1000	1.366	0.980	0.500	1.855	1.172

Table 5.7. Descriptive statistics summary of the FIR measurements of the oral anatomical sites of disease and non disease.

The sample size, mean and median values and the inter quartile range of the FIR that were collected from the two groups showed non normal distribution data, therefore Mann-Whitney Rank Sum Test was used to compare between the two groups (disease and non disease).

The value found was P = 0.465, therefore there was not statistically significant difference (p>0.05) between the FIR values of the readings obtained from cases with metabolic disease and non metabolic disease.

Comparison of the FIR values distribution, inter quartile range and the median values of the disease and non disease are shown in figure 5.11.

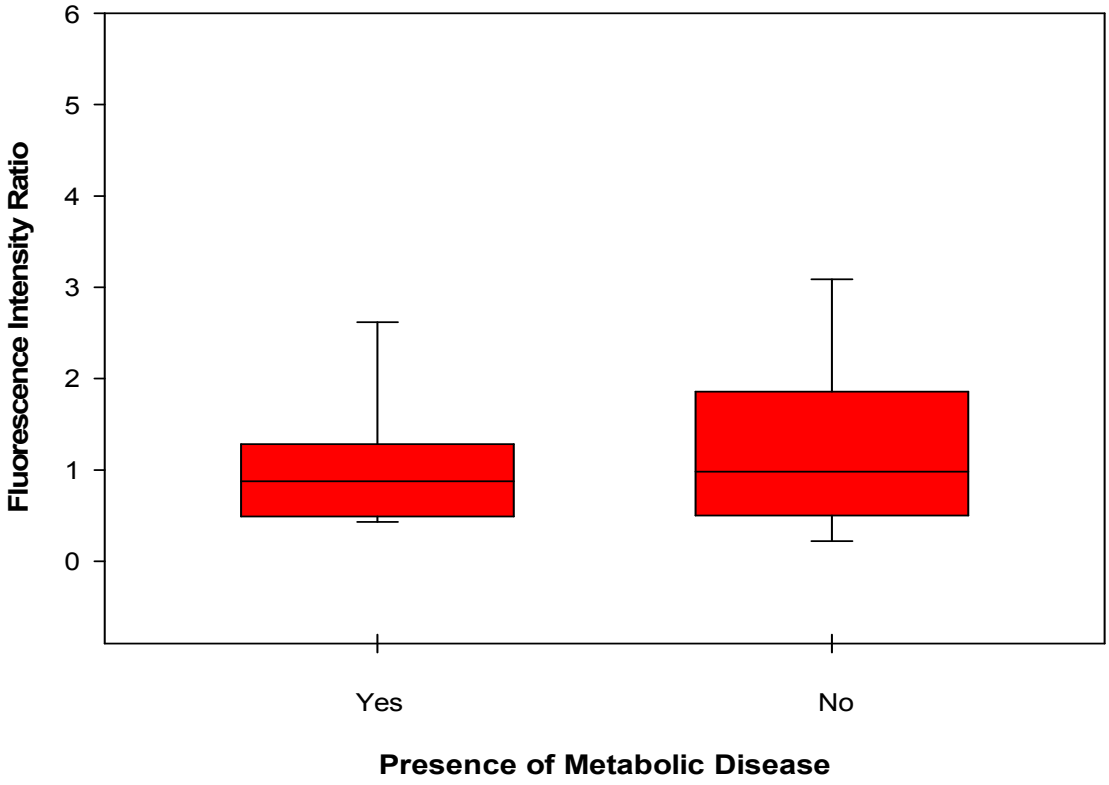


Figure 5.11. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to presence of metabolic diseases.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the presence or absence of systemic or metabolic diseases. They were classified into disease and non disease groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison. It was noticed variation in the peak intensities of the autofluorescence and PpIX between the two groups; however the red/green ratios of these peaks were relatively parallel as shown in figure 5.12.

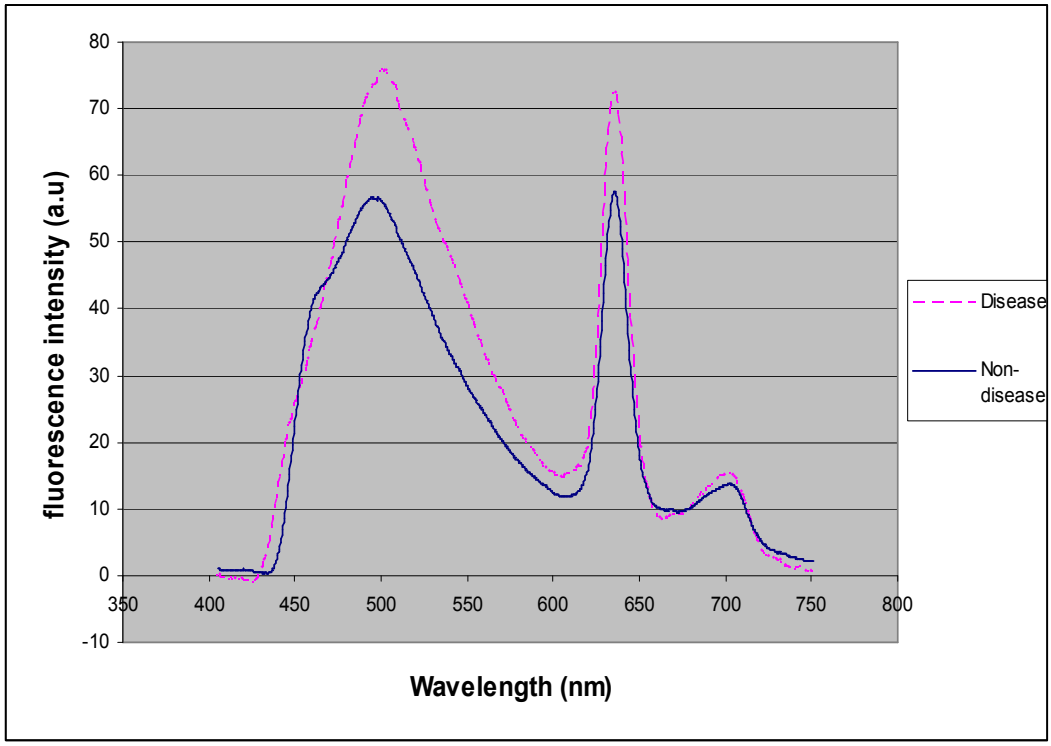


Figure 5.12. Average fluorescence spectra of all oral anatomical sites in relation to the presence of metabolic diseases.

5.2.5 Comparison of FIR measurements of the oral anatomical sites in relation to smoking

The information was collected from the patient information sheet about smoking habit regardless of the number of cigarette per day. They were divided and classified into two groups; smoker and non smoker.

The FIR measurements obtained from all the anatomical sites of the smokers and the non smokers was compared statistically.

A total of 218 readings (FIR) were analysed statistically obtained from 24 patients. They were 116 readings obtained from 12 patients who were smoking cigarette and 102 readings obtained from 12 non smokers.

The cases that had smoking habit were indicated as smoker, while the others as non smoker in the summary table 5.8.

Status	No of Patients	No of FIR Readings	Max	Min	Mean	Median	25%	75%	Std Dev
Smoker	12	116	5.610	0.1000	1.387	1.045	0.505	1.855	1.222
Non Smoker	12	102	4.240	0.120	1.265	0.920	0.500	1.520	1.017

Table 5.8. Descriptive statistics summary of the FIR measurements of the oral anatomical sites of smokers and non smokers.

There was very slight difference in the mean and median values which indicates skewed and non normal data distribution. The difference in the median values between the two groups was assessed using Mann-Whitney Rank Sum Test. We have found the probability was (P = 0.727) which indicates that there was no significant difference (p>0.05) between the FIR of the smoker and non smoker readings.

Comparison of the FIR values distribution, inter quartile range and the median values of the smokers and non smokers are shown in figure 5.13.

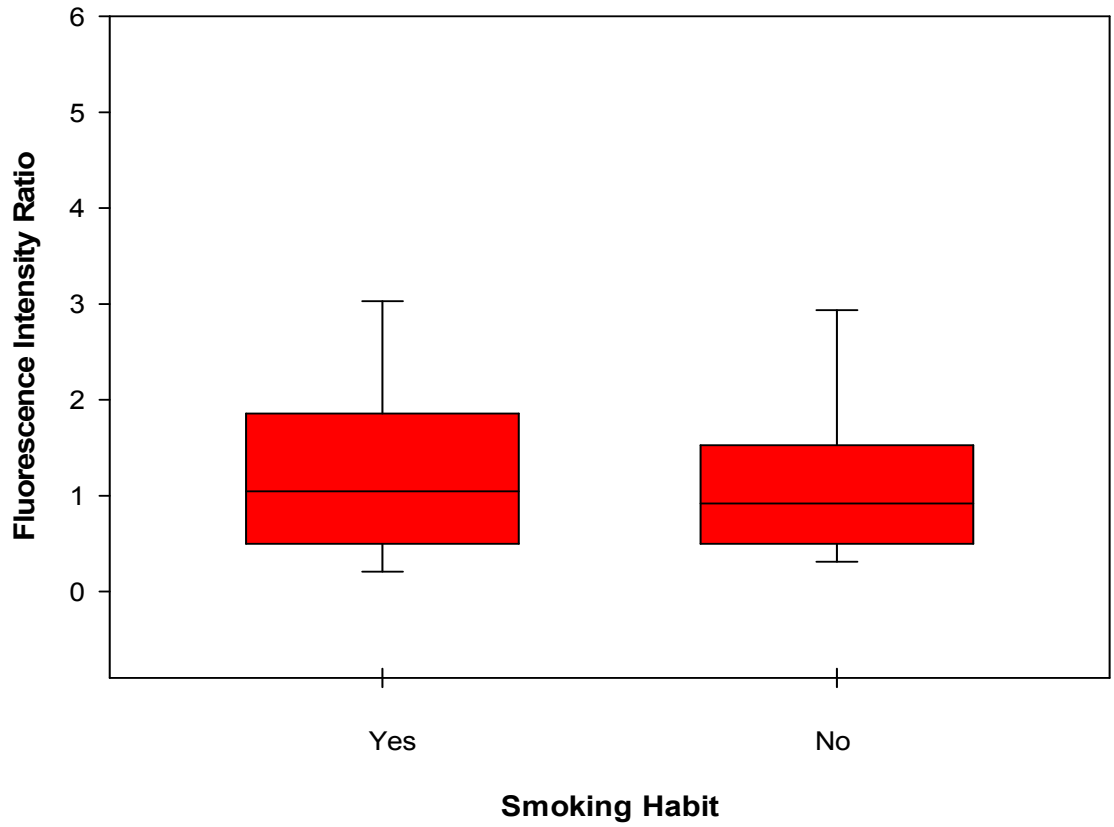


Figure 5.13. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to smoking habit.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the presence or absence of smoking habit. They were classified into smokers and non smokers groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison. The autofluorescence peaks look the same with slight elevation in the smokers PpIX peak as shown in figure 5.14.

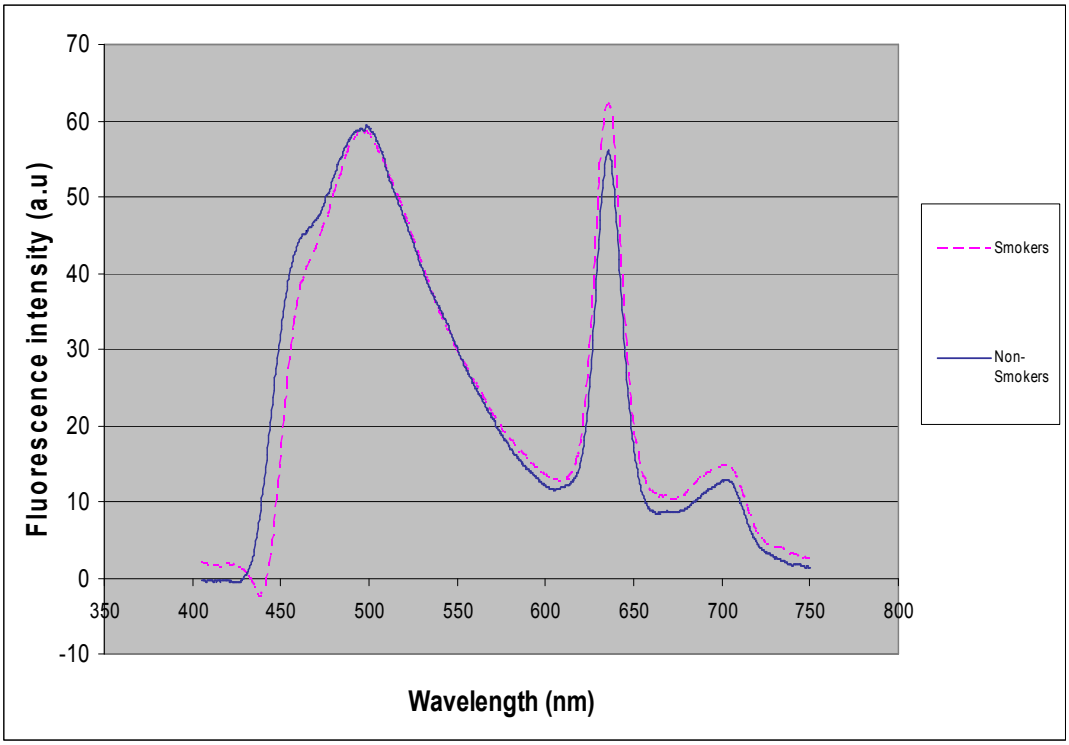


Figure 5.14. Average fluorescence spectra of all oral anatomical sites in relation to the presence of smoking habit.

5.2.6 Comparison of FIR measurements of the oral anatomical sites in relation to alcohol drinking

The information was collected from the patient information sheet about alcohol drinking regardless of the number of cigarette per day. They were divided and classified into two groups; uses of alcoholic and non uses of alcohol.

The FIR measurements obtained from all the anatomical sites of the alcoholic and the non alcoholic were compared statistically.

A total of 218 readings (FIR) were analysed statistically obtained from 24 patients. They were 126 readings obtained from 13 patients who were alcohol users and 92 readings obtained from 11 non alcohol users.

The group that used alcohol was indicated as alcohol, while the other as non alcohol in the summary table (table 5.9).

Status	No of Patients	No of FIR Read	Max	Min	Mean	Median	25%	75%	Std Dev
Alcohol	13	126	5.610	0.1000	1.424	1.020	0.550	1.910	1.233
Non alcohol	11	92	5.570	0.150	1.202	0.960	0.475	1.505	0.964

Table 5.9. Descriptive statistics summary of the FIR measurements of the oral anatomical sites of alcohol users and non alcoholic users.

Slight differences were observed in the mean and median values which indicate skewed and non normal data distribution.

The difference in the median values between the two groups was assessed using Mann-Whitney Rank Sum Test. The analysis showed that the probability was P =0.344, which indicates that there was no significant difference (p>0.05) between the FIR of the alcohol and non alcohol users of the oral anatomical site readings.

Comparison of the FIR values distribution, inter quartile range and the median values of the alcohol and non alcohol groups are shown in figure 5.15.

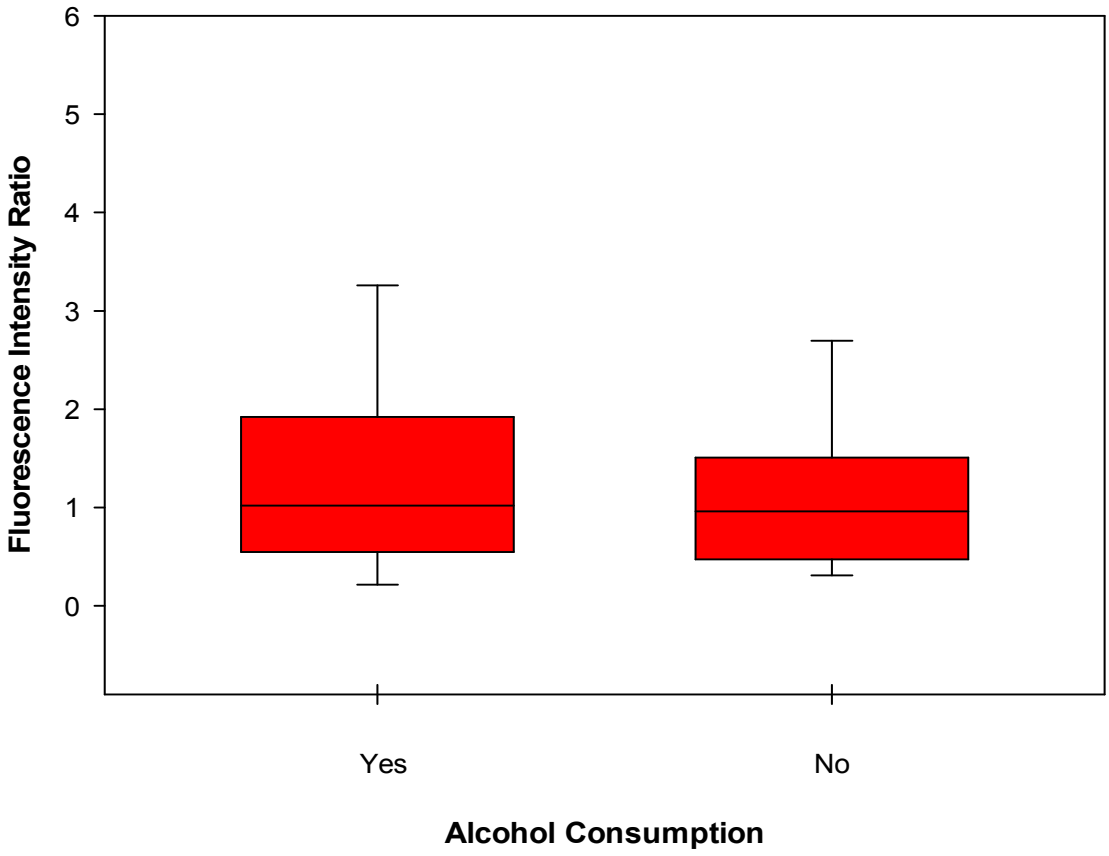


Figure 5.15. Fluorescence intensity ratio at (635/500nm) or red/green ratio in relation to alcohol consumption.

The spectra of all oral anatomical sites obtained from the sample size were averaged in relation to the presence or absence of alcohol consumptoion. They were classified into alcohol and non alcohol groups and plotted in two line graphs to show the spectral lines distribution in one plot for the comparison. The autofluorescence peaks look slightly elevated in non alcohol group while the PpIX peaks were the same level of the two groups as shown in figure 5.16.

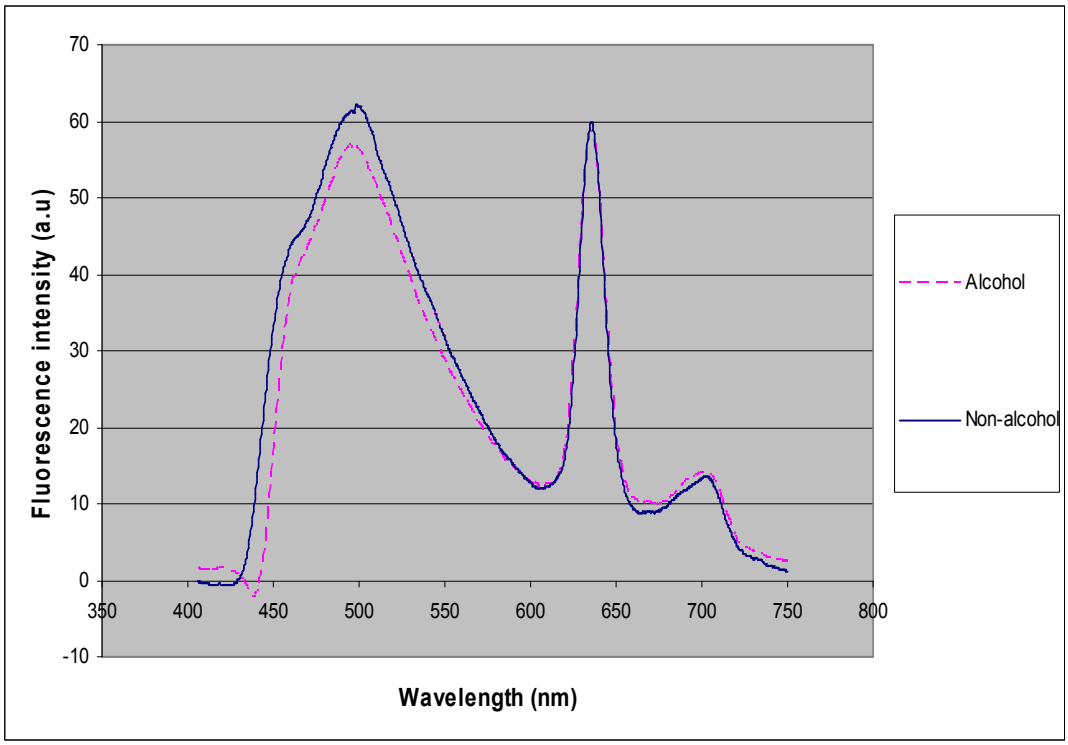


Figure 5.16. Average fluorescence spectra of all oral anatomical sites in relation to alcohol consumption.

5.3 Fluorescence intensity ratio (FIR) measurements in

detecting the oral lesions

A total of 134 spectral readings were obtained from 47 surgically biopsied areas taken from 35 patients for this part of the trial and included in the result analyses. Three PDD readings (3, 34, and 35) and some lesion and normal sites (see appendix 7 table Index 7.6&7) were not obtained because of management difficulties and/or technical errors.

As mentioned previously, the method of collecting the data which were tabulated by including all the readings obtained from the biopsy areas and listed in two columns of readings (table Index 7.6), that was because two readings were obtained from most of the lesions which were surgically punch biopsied. The table showed the number of the biopsies obtained from each patient, site of the biopsy, histopathological diagnosis and the fluorescence intensity levels at 500nm and 635nm and their ratios (red/green ratio) for the lesion and normal sites. The measurements of PDD001-27 were done in Dundee and PDD028-35 in Glasgow.
Table Index 7.7 (appendix 7) showed the approximated values of the fluorescence intensity ratios that were used for the statistical analysis.
Readings not obtained from the patients or some biopsies sites because of technical errors or management difficulties were listed as NO.

5.3.1 Detecting the lesion and normal sites

All the lesion site readings and the normal site readings (more than 5mm away from the lesion) obtained were compared statistically to detect the reliability of the OBS and 5-ALA in the detection of the clinically suspicious oral lesions in general.

Two methods of comparison were followed between the lesions and normal sites. They were:

1-Comparison between the 1st readings, 2nd readings and normal readings. This method was conducted to assess the reliability of the two readings obtained from the lesions and the normal sites for the detection of the oral lesions.

The mean, median, standard deviation and other values were listed in the summary table 5.10.

Site	No of FIR Read	Max	Min	Mean	Median	25%	75%	Std Dev
Lesion 1st Reading	46	15.000	0.0900	1.885	1.290	0.670	2.430	2.331
Lesion 2nd Reading	45	7.090	0.120	1.730	1.360	0.680	2.275	1.483
Normal Reading	43	2.120	0.0900	0.802	0.690	0.310	1.108	0.557

Table 5.10. Descriptive statistics summary of the 1st, 2nd lesion and normal sites FIR.

The three groups of readings were compared using one way analysis of variance. The FIR data were non-normally distributed, therefore Kruskal-Wallis One Way

Analysis of Variance on Ranks test was applied to compare between the groups (the three readings)

The differences in the median values among the three groups was great (H = 15.507 with 2 degrees of freedom) so there was a statistically significant difference (P = <0.001) between the lesion readings and normal site.

Using Dunn's Method for all Pairwise Multiple Comparison Procedures, there was statististically non-significant difference between the 1st and 2nd readings, while there was a statistically significant difference between each of the lesion readings (1st and 2nd) when compared with the normal site readings as shown in table 5.11.

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Lesion 2nd Reading vs Normal Reading	29.263	3.534	Yes
Lesion 2nd Reading vs Lesion 1st Reading	2.035	0.250	No
Lesion 1st Reading vs Normal Reading	27.229	3.306	Yes

Table 5.11. Pairwise comparison between the groups.

Comparison of the readings and the FIR values and the description were plotted in figure 5.17.

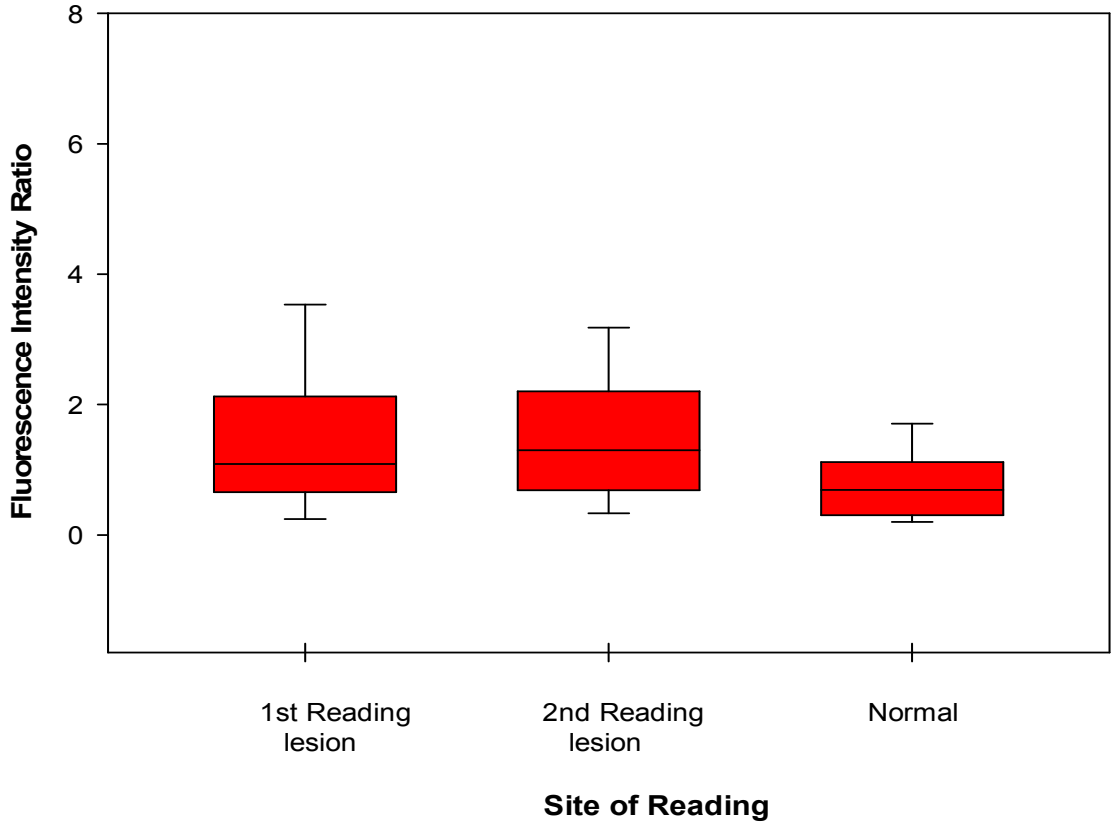


Figure 5.17. Fluorescence intensity ratio at (635/500nm) or red/green ratio of the 1st and 2nd Lesion and normal site readings.

To compare the measurements spectrally, the figures show the average of each sample size obtained (1st, 2nd lesion and normal sites) in one line graph. Number of FIR Readings (46, 45 lesion and 43 normal as shown in table 5.8) was averaged to form the three line graphs for the spectra comparison. We drew two graphs, on real time measurements as obtained from the patients (figure 5.18) and the spectra after normalization (transform the data to Logarithm values) to give better spectra comparison interpretation (figure 5.19). The line graphs of the two lesion site measurements and the fluorescence intensity peaks were noticed lower in the autofluorescence (green or 500nm) region when compared with the normal spectra

which was higher. However, the 1st and 2nd lesion readings were at the same level at PpIX (red or 635nm) region (Figure 5.18).

There was a difference in the green region peaks of the 1st and 2nd lesion readings which may affect the red/green ratio (FIR), but the difference was statistically non significant.

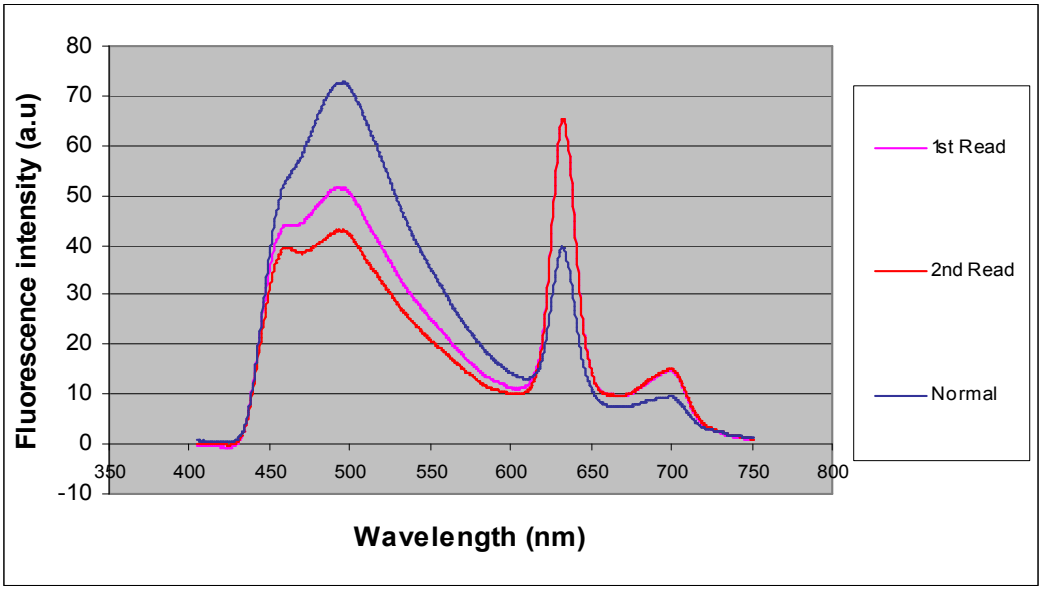


Figure 5.18. Average fluorescence spectra of 46 1st and 45 2nd lesion and 43 normal site readings.

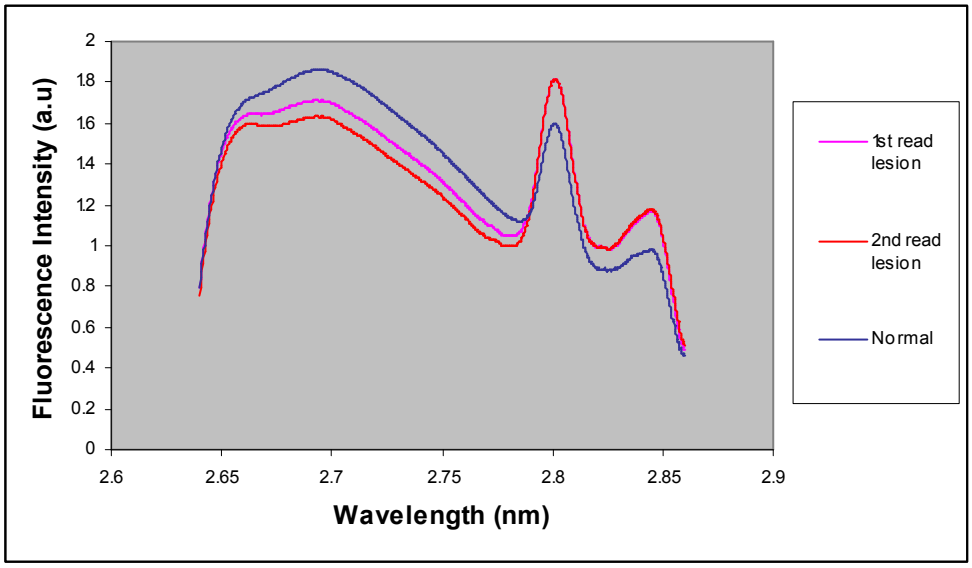


Figure 5.19. Normalization of the average fluorescence spectra of 46 1st and 45 2nd lesion and 43 normal sites.

2- The 2nd method of comparison was conducted between lesion sites and normal sites using the same sets of readings.

The 1st and 2nd readings obtained from the lesion sites, which consisted of all types of lesions, were merged together to form one single group (91 readings) and indicated in the tables and figures as lesion sites. These readings were compared with the normal sites (43 readings) and categorized into two groups, lesion sites (1st & 2nd readings combined) and normal sites.

Descriptive statistic of the FIR levels were calculated and summarized in table 5.12.

Site	No of FIR Read	Max	Min	Mean	Median	25%	75%	Std Dev
Lesion Sites	91	15.000	0.0900	1.808	1.350	0.680	2.305	1.949
Normal Sites	43	2.120	0.0900	0.802	0.690	0.310	1.108	0.557

Table 5.12. Descriptive statistics summary of FIR readings at the lesion and normal sites

The results showed that the mean values were noticeably higher than the median values of the FIR of the lesion and normal sites, this indicates that the distribution of the data were skewed and non normal in distribution at both sites.

There was a noticeable difference between the minimum and maximum values which indicate variation in the data obtained from the lesion sites. Dispersion of the data was also indicated by the high standard deviation which emphasis the variation in cellular fluorescence emission or FIR values of the different oral lesion types, it was also noticeable in comparison with the normal sites. Furthermore, it was also indicated by the inter quartile range.

The inter quartile range of the lesion site shows the data were taken from different kinds of oral lesions (hyperkeratosis, inflammatory lesions, and dysplastic lesions) which interpret the variation of the FIR values obtained from these locations as plotted in figure 5.20.

Comparison between the two readings in non normal data distribution, Mann-Whitney rank sum test was recommended to detect the probability. The analysis

showed that the statistically significant difference in the median values between the two groups rejected the null hypothesis that there was no difference between the fluorescence of the lesion and normal (Mann-Whitney U Statistic= 2781.000, T = 2078.000); therefore, there was a highly significant difference between the lesion and normal site readings (P = <0.001).

Comparison and the distribution of the data were shown in figure 5.20.

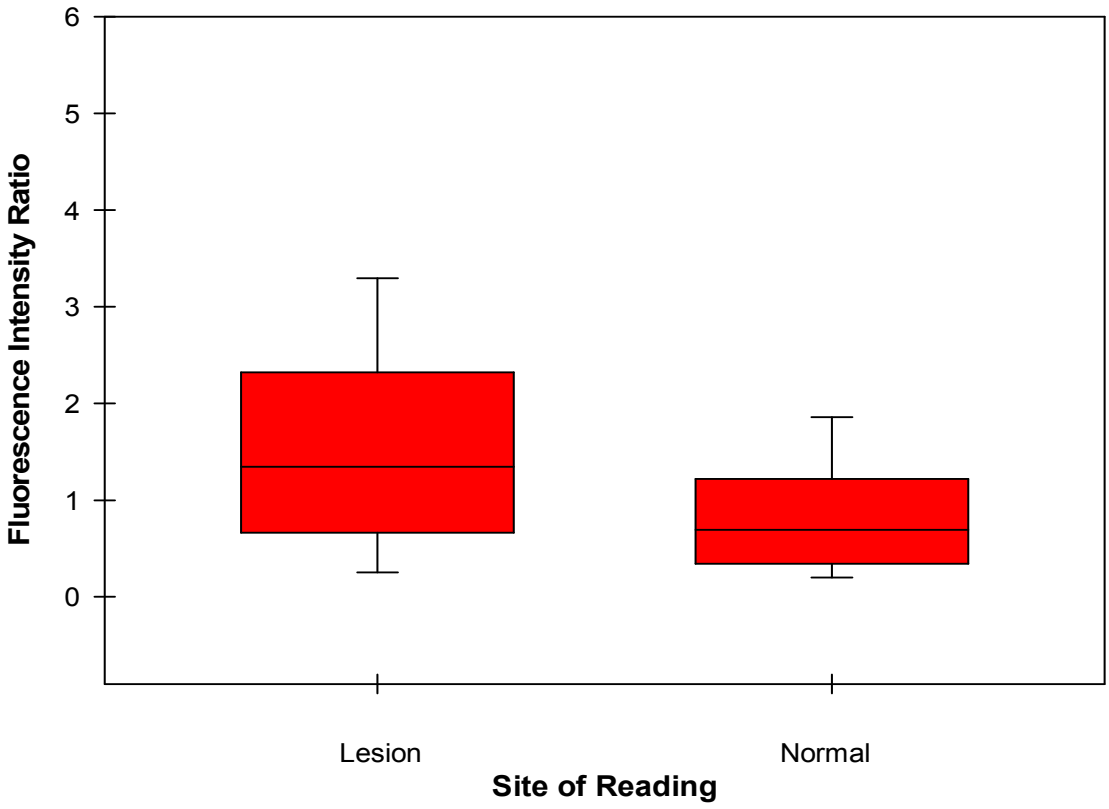


Figure 5.20. Fluorescence intensity ratio at (635/500nm) or red/green ratio of the lesion and normal site readings.

The box plot graphs shows the data distribution, the inter quartile range, and median values. The total sample size of the lesion (91) and normal (43) spectra as

shown in table 5.10 were averaged to draw the two line graphs of the lesion and normal site readings for comparison.

The spectra graphs show the variability in the fluorescence intensity peaks at the autofluorescence (green or 500nm) and PpIX (red or 635nm) regions. Both real time measurements and spectra after normalization show the difference which the red/green ratios (FIR) were statistically significant as shown in figures 5.21 and 5.22 respectively.

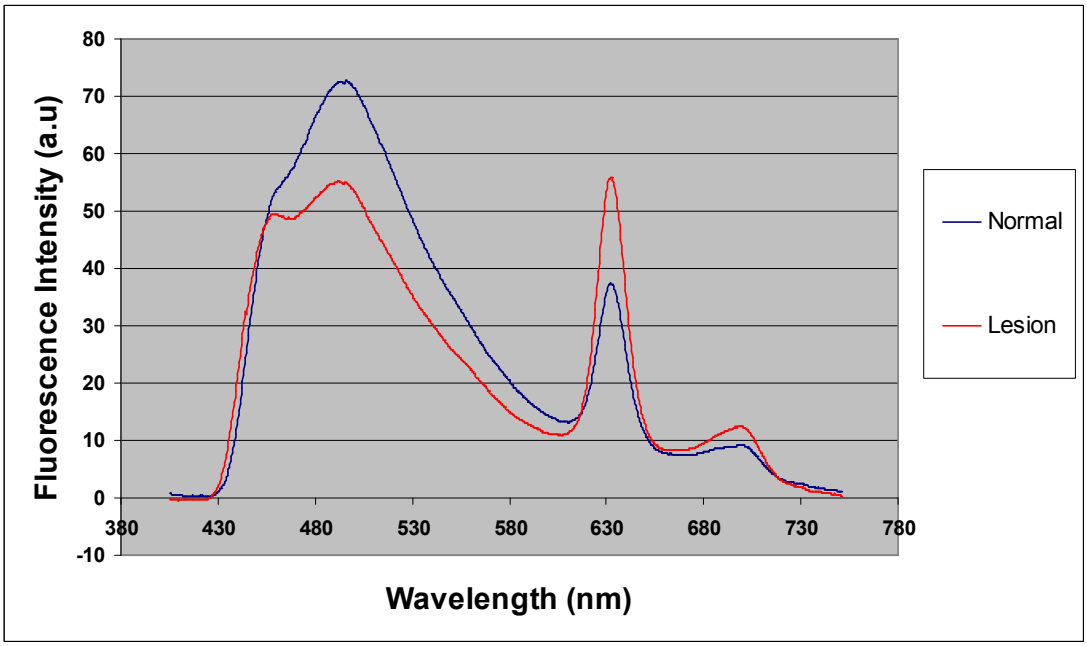


Figure 5.21. Average fluorescence spectra of 91 lesion and 43 normal sites

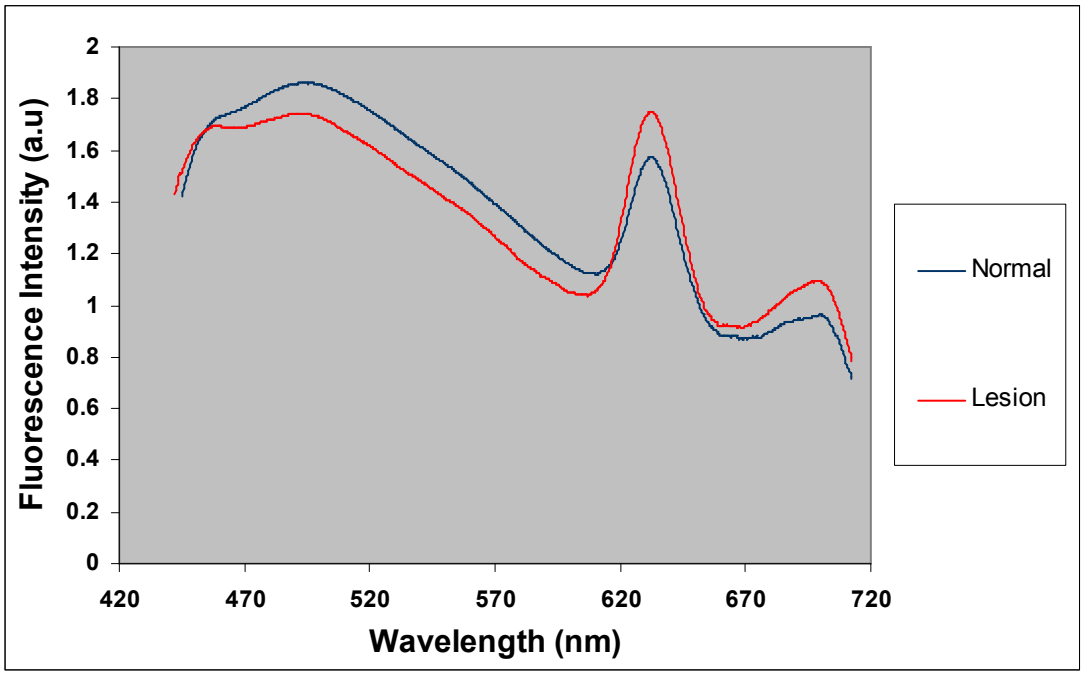


Figure 5.22. Normalization of the average fluorescence spectra of 91 lesion and 43 normal sites.

5.3.2 Detecting clinically suspicious oral lesions

The same set of readings (FIR) were classified according to the biopsy results and analysed statistically.

Neither the investigators, who took the fluorescence measurement nor the pathologist, who wrote the reports were aware of each other outcome i.e. they were blinded to the findings. The values of the FIR were classified into three groups according to the microscopic reports obtained from the pathologist who diagnosed the biopsies microscopically.

The classification was based on the following microscopic findings;

1- Hyperkeratotic; Lesions show thickness of the keratin layer (parakeratosis or orthokeratosis) with no evidence of any inflammatory cell infiltrate of the submucosa (lamina properia) or superimposed with candidiasis.
2- Inflammatory lesions: Keratotic and non keratotic lesions showed evidence of band of inflammatory or lymphocytic infiltration in the submucosa (Lichenoid reaction and lichen planus) and /or superimposed with candidiasis.
3- Dysplasia; cellular atypia, severe dysplasia and squamous neoplastic changes.

FIR readings of the diagnosed lesions were compared with one another and with the normal site readings. Summary table (table 5.13) showed sample size (No of FIR Read) obtained from each location and the descriptive statistics.

Site	No of FIR Read	Max	Min	Mean	Median	25%	75%	Std Dev
Normal	43	2.120	0.0900	0.802	0.690	0.310	1.108	0.557
Hyperkeratotic	26	15.000	0.0900	1.757	1.180	0.460	2.080	2.834
Inflammatory lesion	47	5.340	0.130	1.644	1.100	0.625	2.365	1.469
Dysplasia	18	7.090	0.850	2.309	1.890	1.350	3.100	1.456

Table 5.13. Summary of the FIR measurements descriptive statistics of the groups (type of the lesions).

Since the data were unequal in size and not normally distributed Kruskal-Wallis One Way Analysis of Variance was used to compare between the groups.

It was found the differences in the median values among all the groups were great (H = 23.864 with 3 degrees of freedom) enough to reject the null hypothesis (P = <0.001) so that there was a highly statistically significant difference between all sites (all types of lesion and normal site readings).

Dunn's Method was applied in All Pairwise Multiple Comparison Procedures to compare between the two groups.

Comparison	Diff of Ranks	Q	P<0.05
Dysplastics vs Normal	51.647	4.738	Yes
Dysplastics vs Hyperkeratotic	32.299	2.713	Yes
Dysplastics vs Inflammatory	27.462	2.552	No
Inflammatory lesion vs Normal	24.185	2.952	Yes
Inflammatory vs Hyperkeratotic	4.838	0.510	No
Hyperkeratotic vs Normal	19.347	2.006	No

Table 5.14. Pairwise comparison between the groups (type of the lesions).

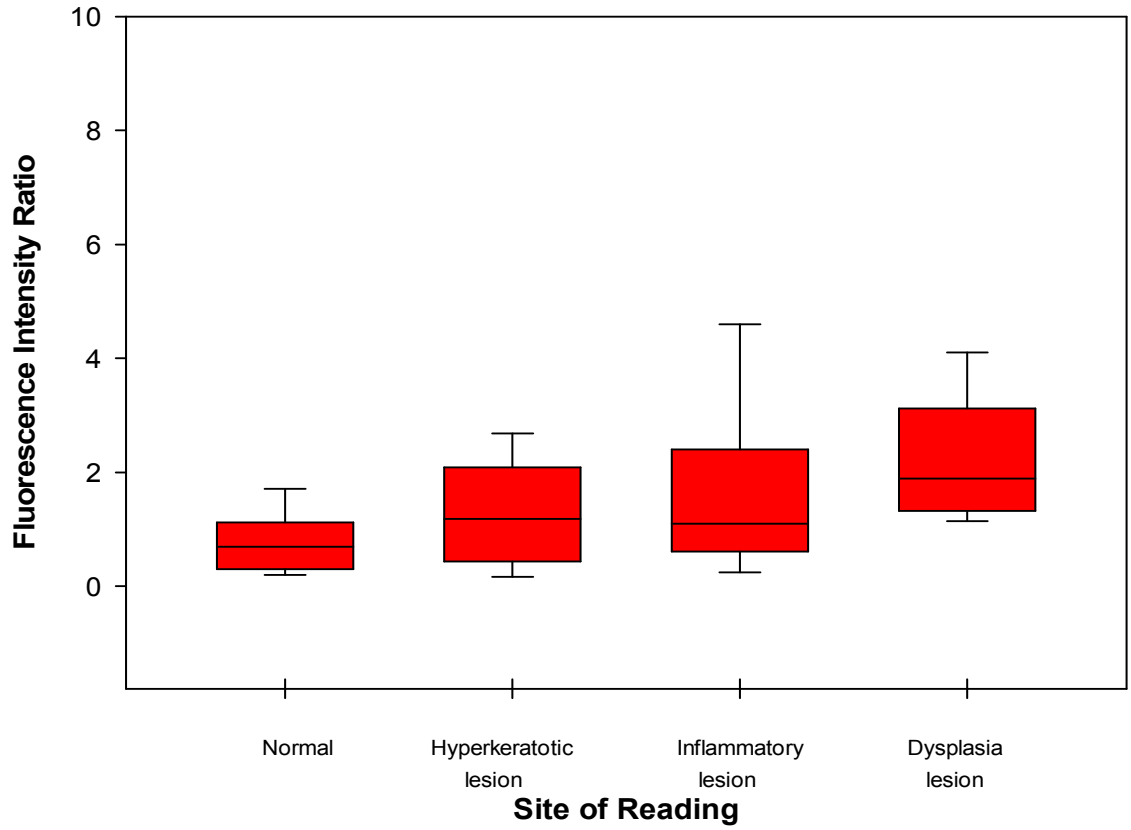


Figure 5.23. Fluorescence intensity ratio at (635/500nm) or red/green ratio of the diagnosed oral lesions and normal sites.

The total sample size of each type of diagnosed oral lesion and normal site spectra were averaged to display the shape of the line graphs and show the autofluorescence and PpIX peaks.

A total of 43 normal, 26 benign hyperkeratotic, 47 inflammatory and 18 dysplasia spectra were averaged in relation the histology classification group and displayed into four line graphs as shown in figure 5.24.

The autofluorescence intensity (green spectrum) of the normal site spectra is the highest peak followed by benign hyperkeratotic, inflammatory and dysplasia lesions respectively. However the PpIX (red) peak was lowest in normal site spectra and increases in the lesion sites. There was noticeable overlap between inflammatory and dysplasia PpIX peaks as shown in the spectra graphs (figure 5.24 the real time averaged spectra and figure 5.25, spectra after normalisation or transformed data).

The spectra graphs show the variability in the fluorescence intensity peaks at the autofluorescence (green or 500nm) and PpIX (red or 635nm) regions. This comparison was noticed both in real time spectra measurements and after normalization. This finding comes in consistent with the results of the statistical analysis presents in table 5.14.

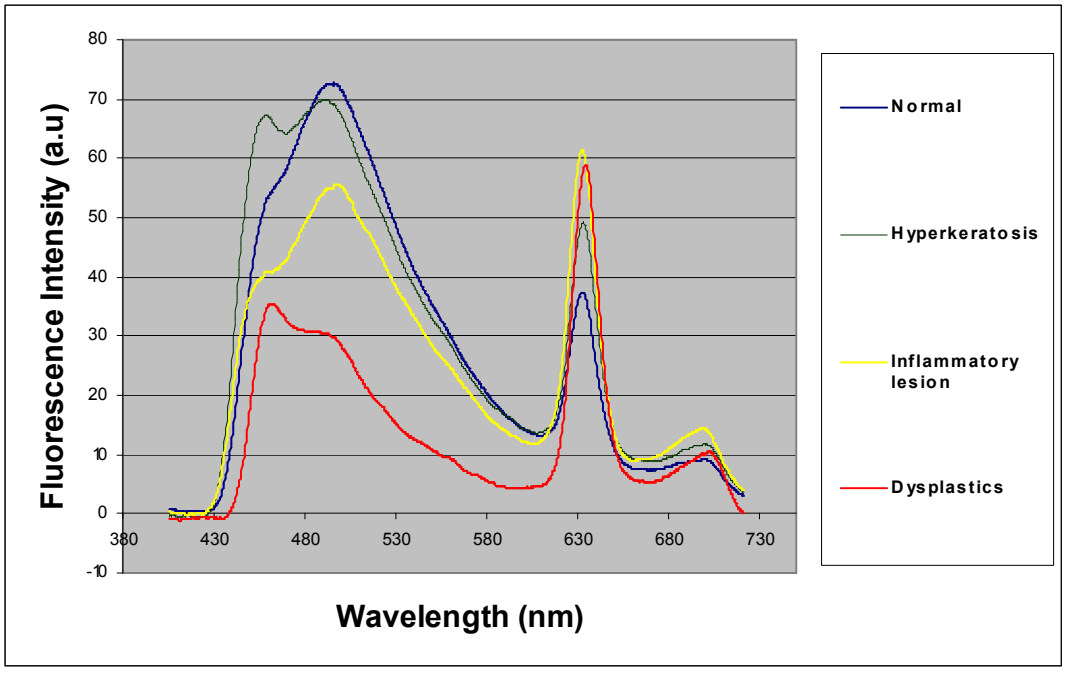


Figure 5.24. Averages of the fluorescence spectra obtained from 43 normal, 26 hyperkeratotic lesion, 47 inflammatory lesion and 18 dysplastic lesion sites.

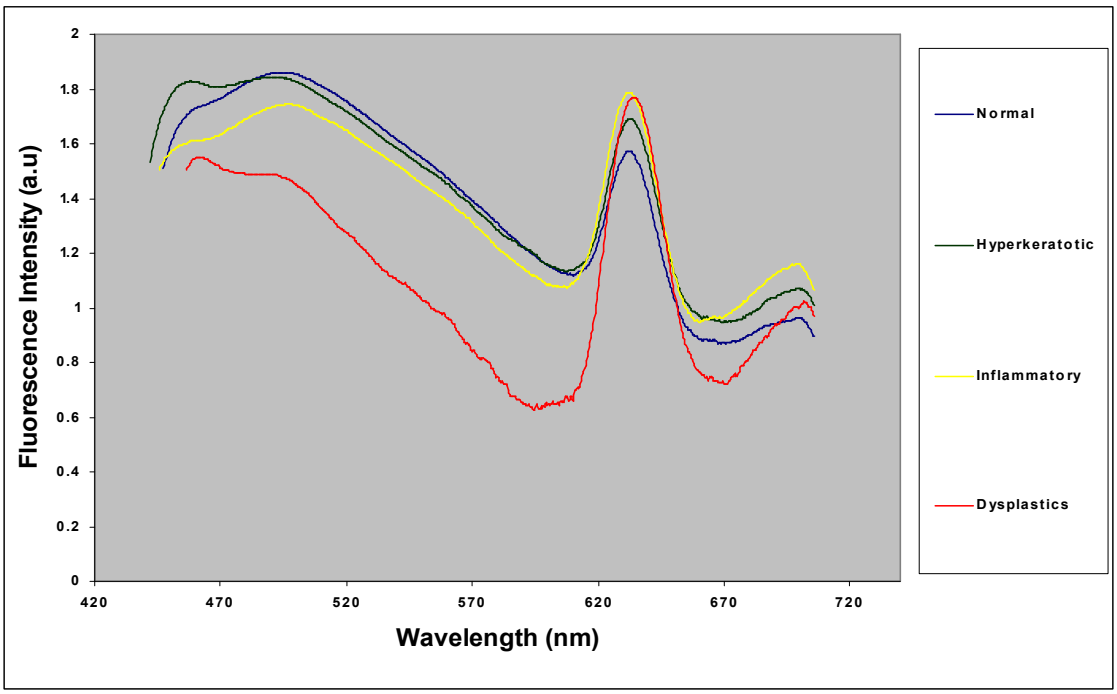


Figure 5.25. Normalized of the average fluorescence spectra obtained from 43 normal, 26 hyperkeratotic lesion, 47 inflammatory lesion and 18 dysplastic lesion sites.

5.3.3 Sensitivity and specificity of the use Fluorescence

intensity ratio (FIR) measurements in detecting oral premalignant (clinically suspicious) lesions

The data in appendix 7 table Index 7.7 were used for this part of the result section. Fluorescence intensity ratios of the diagnosed lesions and normal sites were utilized to detect the sensitivity and specificity of fluorescence in assessing clinically suspicious oral lesions.

5.3.3.1 Sensitivity and specificity of FIR in detecting oral lesions

For optimal data analysis, FIR should be obtained from normal and abnormal sites in each patient. Since the technique of data collection was two readings obtained from the lesion sites and one from the normal site (more than 5mm away from the lesion border), it would be wise to compare the 1st readings only of the lesions regardless of the diagnosis with the readings obtained from the normal sites. This occurred in 43 biopsy site since the normal readings from four of the biopsied patients were not obtained (biopsy 2, 3, 39, and biopsy 42 see table Index 7.6&7).
Figure 5.26 shows the FIR of the 1st lesion and normal site readings of 47 biopsies. Biopsy no. 2,3,39 and 42 showed missing normal reading, hence will not be included in the analysis. The data from these sites were not used in analyzing the sensitivity and specificity of FIR in detecting oral lesions, therefore the 1st readings of a total of 42 biopsy site readings were only included, therefore a total of 84 measurements were included for the analysis.

The FIR of the histologically abnormal (lesion) sites were greater than those of the normal sites except for the readings from biopsies no. 6, 11 and 41 which were readings taken from the tongue and floor of the mouth. Because of considerable

patient –to– patient variation in FIR values, the separation between abnormal and normal could be better visualized when the abnormal/normal ratio were calculated to obtain the cutoff value.

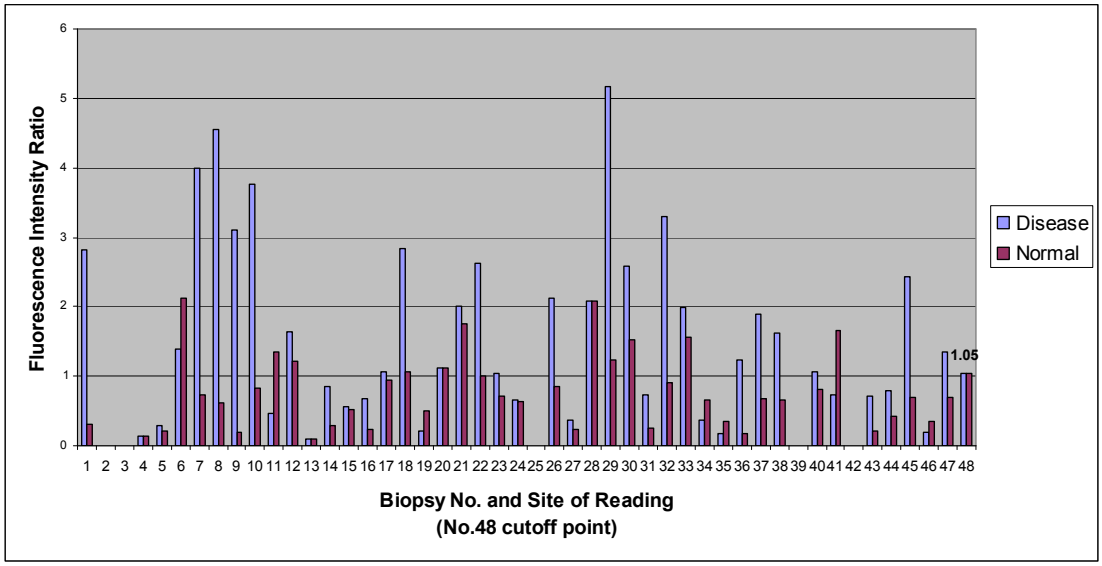


Figure 5.26. Comparison of the FIR values of the lesion and normal site readings. Despite patient-to-patient variability, it is clearly observed the disease site readings (blue bars) are greater than the normal site readings. Data from biopsy 2, 3, 25, 39 and 42 were missing and No. 48 representing the cutoff point (1.05).

The cutoff points were obtained by calculating the standard deviation of all groups to discriminate between the upper and lower values.

One Standard Deviation of the total of normal and lesion readings was 1.05 and considered cutoff point as shown in figure 5.26.

If the FIR value of the clinically identified lesion was greater than the cutoff point then the spectroscopic reading was considered true positive. Otherwise it was classified as false negative. If the FIR value obtained from the normal site was less than the cutoff point, then considered as true negative. Otherwise, if above 1.05 it

was considered as false positive. Using this value (1.05) as a cutoff point to classify the FIR readings into true positive and negative results was shown in table 5.15.

Site	Positive FIR	Negative FIR	Total
Lesion	25	17	42
Normal	11	31	42
Total	36	48	

Table 5.15. Sensitivity and specificity of FIR value in detecting oral lesions.

By following this criterion the sensitivity yielded was 59.5% (true positive/true positive + false negative, 25/42) and specificity was 73.8% (true negative/true negative + false positive 31/42). The positive predictive value (PPV) was 69.4% (true positive TP / (TP + FP) total positives, 25/36) and negative predictive value (NPV) was 65% (true negative TN / (FN + TN) total negatives, 31/48).

5.3.3.2 Sensitivity and specificity of FIR in detecting potentially malignant oral lesions

To determine the sensitivity and specificity of the use of FIR measurements in detecting the potentially malignant (clinically suspicious) oral lesions and their normal site, three comparisons were conducted;

5.3.3.2.1 Comparison between FIR measurements of dysplasia lesions and the normal sites

The 1st readings of eight histologically diagnosed dysplasia lesions and normal (more than 5mm away from the lesion) i.e. total of 16 measurements were utilized for the analysis, since normal site FIR measurement was not obtained from patient no. 28* (see appendix 7 table Index 7.7) so the dysplasia reading was not included.

The same method of analysis was followed by taking one standard deviation of the 1st reading of the lesion and normal sites and considered as a cutoff point to discriminate between the two groups. If the FIR value of the dysplasia lesion was greater than the cutoff point then the spectroscopic reading was considered true positive. Otherwise it was classified as false negative. If the FIR value obtained from the normal site was less than the cutoff point, then considered as true negative. Using this value (1.127) as a cutoff point to classify the FIR readings into true positive and negative was shown in table 5.16.

Figure 5.27 showed the FIR measurements obtained from eight dysplasia and normal sites and the cutoff point. It was observed that all the FIR measurements of dysplasia were greater than that of the normal sites. Cutoff point was noticed higher than all the normal measurements and lower than that of dysplasia as shown in figure 5.27.

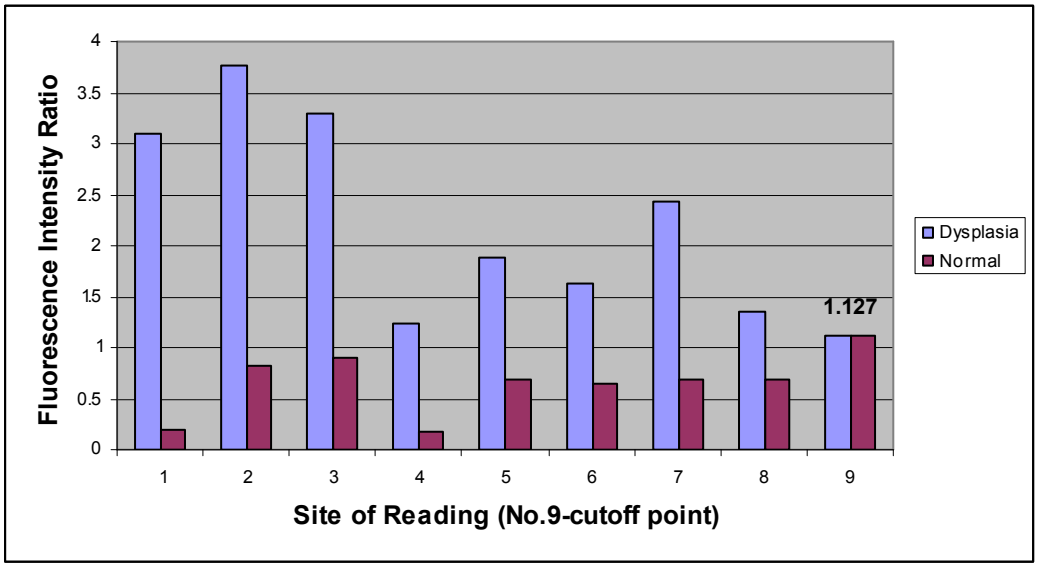


Figure 5.27. Comparison of the FIR measurements of the dysplasia and normal sites.

By following this criterion the sensitivity yielded was 100 % (true positive/true positive + false negative, 8/8) and specificity was 100% (true negative/true negative + false positive, 8/8) as shown in table 5.16. The PPV was 100% (true positive TP / (TP + FP) total positive, 8/8) and the NPV was 100% (true negative TN / (FN + TN) total negative, 8/8)

Site	Positive FIR	Negative FIR	Total
Dysplasia	8	0	8
Normal	0	8	8
Total	8	8	

Table 5.16. Sensitivity and specificity of FIR value in discriminating between dysplasia and normal sites.

5.3.3.2.2 Comparison between FIR measurements of inflammatory

lesions and the normal site readings

The 1st readings of twenty four biopsied inflammatory lesions and their normal (more than 5mm away from the lesion border) sites were used for this part of study. A total of 44 measurements were utilized for the analysis, since normal site FIR measurements were not obtained from patient no. 1 (biopsy 2) and patient no.30 (biopsy 2) (see appendix 7 table Index 7.7) so the inflammatory readings of these readings were not included.

The same method of analysis was followed by taking the value of one standard deviation of all the 1st reading of the lesion and normal sites and considered as a cutoff point to discriminate between the two groups.

If the FIR value of the inflammatory lesion was greater than the cutoff point then the spectroscopic reading was considered true positive (statistically). Otherwise it was classified as false negative. If the FIR value obtained from the normal site was less than the cutoff point, then considered as true negative. Otherwise it was classified as false positive.

Figure 5.28 has shown the FIR measurements obtained from the inflammatory lesions, normal sites and the cutoff point. It was observed that the FIR measurements of 10 inflammatory lesions were greater than the Cutoff point hence considered true positive and seven of the normal sites considered false positive.

The values of 15 normal readings were (below the cutoff point) considered true negative and 12 inflammatory readings considered false negative.

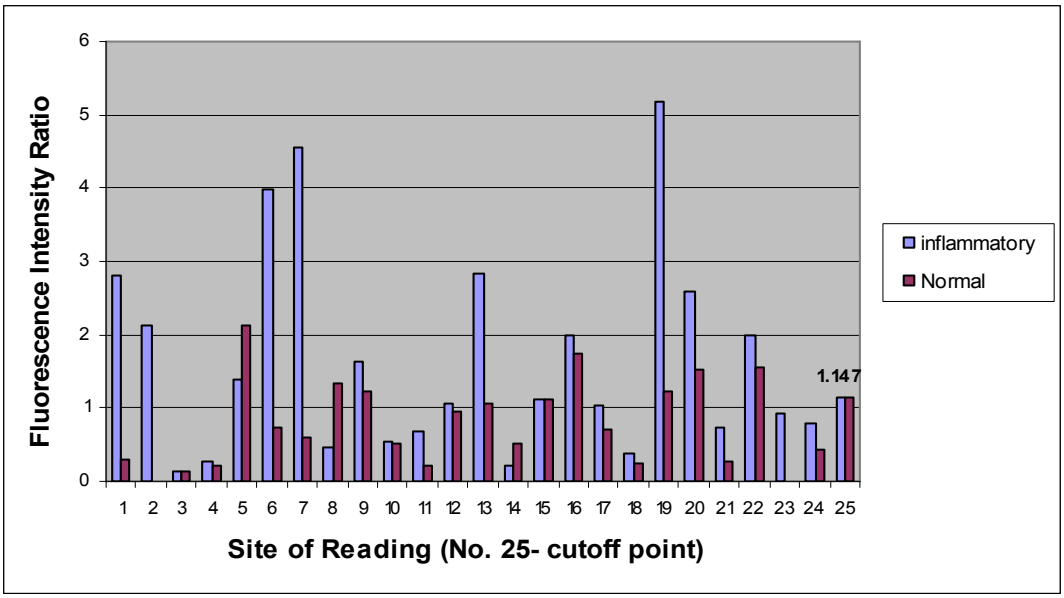


Figure 5.28. Comparison of the FIR measurements obtained from the inflammatory lesions and normal sites.

By following this criterion the sensitivity yielded was 45% (true positive/true positive + false negative, 10/22) and specificity was 68% (true negative/true negative + false positive, 15/22) as shown in table 5.17. The PPV was 58% (true positive TP / (TP + FP) total positive, 10/17) and NPV was 55% (true negative TN / (FN + TN) total negative, 15/27)

Site	Positive FIR	Negative FIR	Total
Inflammatory lesion	10	12	22
Normal	7	15	22
Total	17	27	

Table 5.17. Sensitivity and specificity of FIR value in discriminating between inflammatory lesions and normal sites.

5.3.3.2.3 Comparison between FIR measurements of lichen planus and lichenoid lesions with the normal sites

The 1st readings of eighteen histologically diagnosed lichen planus (Lp) and lichenoid lesions (LL) and normal (more than 5mm away from the lesion border) i.e. total of 36 measurements were utilized for the analysis, since normal site FIR measurement was not obtained from patient no. 30* (see appendix 7 table Index 7.7) so the lesion reading was not included.

The same method of analysis was followed by merging the two sets of readings (the 1st reading of the Lp&LL and normal) and taking the value of one standard deviation of readings and considered as a cutoff point to discriminate between the two groups. If the FIR value of the Lp&LL lesion was greater than the cutoff point then the spectroscopic reading was considered true positive. Otherwise it was classified as false negative. If the FIR value obtained from the normal site was less than the cutoff point, then considered as true negative, otherwise was considered false negative.

Figure 5.29 showed the FIR measurements obtained from nineteen Lp&LL, The same number (but one missing value i.e. 18 readings) from the normal sites and the cutoff point. It was observed that the FIR measurements of ten Lp&LL biopsy sites were higher than the cuttoff point and hence considered true positive. While 8 readings obtained from the normal sites were greater than the cuttoff point and considered false positive. Ten of the normal readings were greater than the cuttoff point and considered false negative, while 8 of Lp&LL were less than the cuttoff point and considered true negative for the analysis.

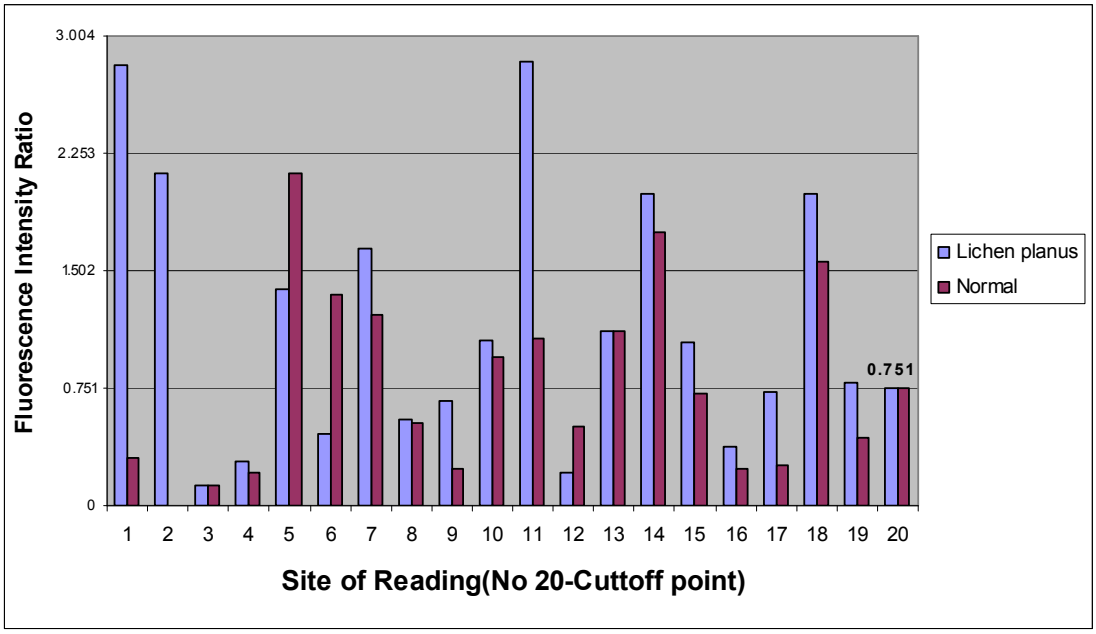


Figure 5.29. Comparison of the FIR measurements of the Lp&LL and normal sites. Biopsy No.2 has no normal reading and was not included.

Using this value (0.751) as a cutoff point to classify the FIR readings into true positive and negative was shown in table 5.18. By following this criterion the sensitivity yielded was 55 % (true positive/true positive + false negative, 10/18) and specificity was 55% (true negative/true negative + false positive, 10/18) as shown in table 5.15. The positive predictive value was 55% (true positive TP / (TP + FP) total positive, 10/18) and negative predictive value was 55% (true negative TN / (FN + TN) total negative, 10/18).

Site	Positive	Negative	Total
Lp&LL	10	8	18
Normal	8	10	18
Total	18	18	

Table 5.18. Sensitivity and specificity of FIR value in discriminating between Lp&LL and normal sites.

5.3.3.3 Comparison between FIR measurements of dysplasia and other oral lesions

To determine the sensitivity and specificity of the use of FIR measurements in detecting dysplasia, the following comparisons were conducted;

5.3.3.3.1 Comparison between FIR measurements of dysplastic and hyerkeratotic lesions

The 1st and 2nd measurements of nine histologically diagnosed dysplasia lesions and the 1st and 2nd readings from the same sample size selected in sequence of benign hyperkeratosis (total of 36 FIR measurements) were included in this analysis (appendix 7 table Index 7.7).

Figure 5.30 showed the FIR measurements of the dysplasia and benign hyperkeratosis histologically diagnosed lesions. Six hyperkeratosis measurements were noticed higher than the cutoff point (false positive), while fourteen dysplasia measurements exceeded the point (true positive).

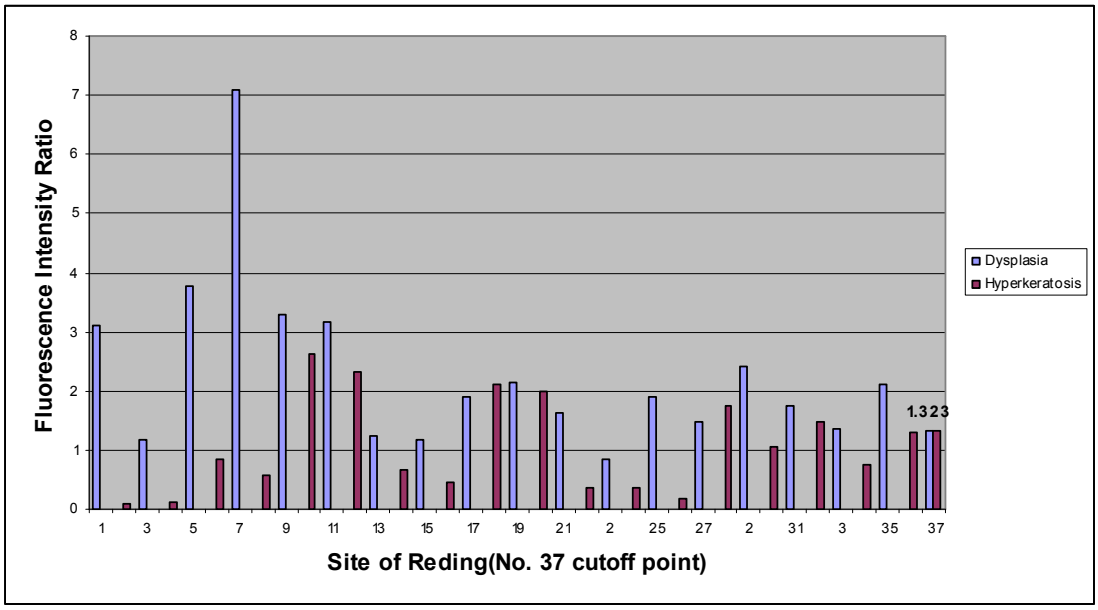


Figure 5.30. Comparison of the FIR measurements of the dysplasia and benign hyperkeratosis.

Using the value of one standard deviation (1.323) as a cutoff point to classify the FIR readings into true positive and negative results is shown in table 5.19, it was noticed that the sensitivity yielded was 77.7% (true positive/true positive + false negative, 14/18) and specificity was 66.6% (true negative/true negative + false positive, 12/18). The PPV was 70% (true positive TP / (TP + FP) total positive, 14/20) and the NPV was 75% (true negative TN / (FN + TN) total negative, 12/16).

Site	Positive FIR	Negative FIR	Total
Dysplasia	14	4	18
Hyperkeratosis	6	12	18
Total	20	16	

Table 5.19. Sensitivity and specificity of FIR value in discriminating between dysplasia and benign hyperkeratosis sites.

5.3.3.3.2 Comparison between FIR measurements of dysplasia and inflammatory (lichen planus and lichenoid) lesions

The 1st readings of nine histologically diagnosed dysplasia lesions and the 1st readings from the same sample size of inflammatory lesions (lichen planus and lichenoid lesion) i.e. total of 36 FIR measurements were randomly selected and included in this analysis (appendix 7 table Index 7.7).

Figure 5.31 shows the FIR measurements of the dysplasia and inflammatory lesions (lichen planus and lichenoid lesion) and the cutoff point (no.19 equal to 1.431). Six of the inflammatory lesions representing lichen planus and lichenoid lesions were noticed higher than the cutoff point (false positive) and twelve were true negative, while thirteen dysplasia lesions were considered true positive and five false negative.

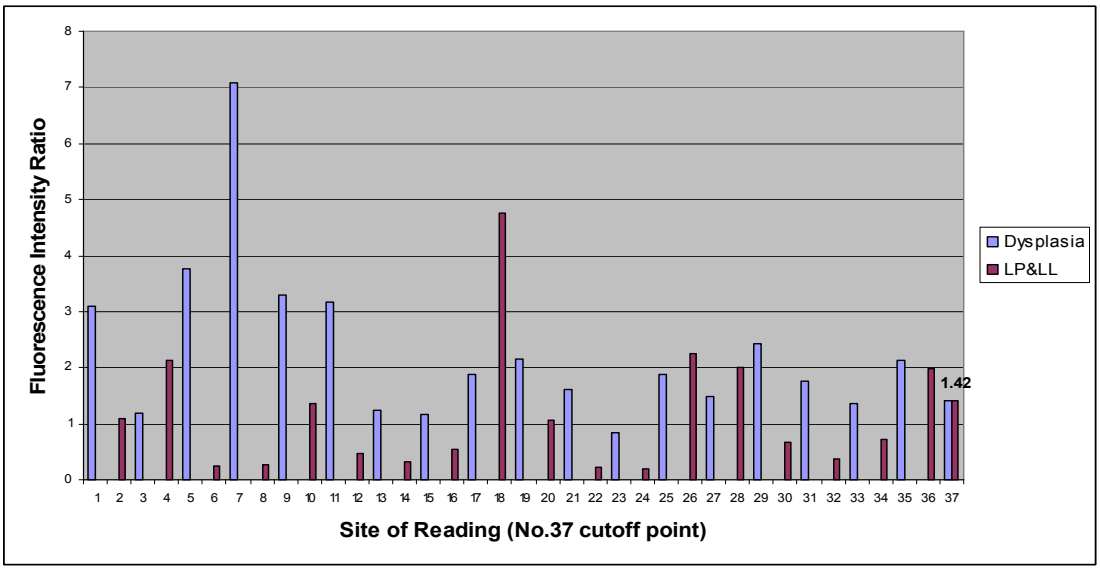


Figure 5.31. Comparison of the FIR measurements of the dysplasia and inflammatory lesions (lichen planus and lichenoid lesions).

Using the value of one standard deviation (1.431) as a cutoff point to classify the FIR readings into true positive and negative results is shown in table 5.20. Six of the inflammatory lesions representing lichen planus and lichenoid lesions were noticed higher than the cutoff point (false positive) and twelve were true negative, while thirteen dysplasia lesions were considered true positive and five false negative.

It was noticed that the sensitivity yielded was 72.2% (true positive/true positive + false negative, 13/18) and specificity was 66.6% (true negative/true negative + false positive, 12/18). The PPV was 68 % (true positive TP / (TP + FP) total positive, 14/20) and the NPV was 70% (true negative TN / (FN + TN) total negative, 12/17).

Site	Positive FIR	Negative FIR	Total
Dysplasia	13	5	18
Lichen planus & Lichenoid Lesion	6	12	18
Total	19	17	

Table 5.20. Sensitivity and specificity of FIR value in Discriminating between dysplasia and inflammatory (lichen planus and lichenoid lesions) sites.

5.3.3.3.3 Comparison between FIR measurements of dysplasia and inflammatory (Candidal leukoplakia) lesions

The 1st readings and 2nd readings obtained from five histologically diagnosed lesions candidal leukoplakia and the same sample size of dysplasia were included for this analysis.

A total of 20 FIR measurements were used to compare between the two groups. Figure 5.32 showed the FIR measurements of the dysplasia and inflammatory lesions and the cutoff point (no. 21 equal to 1.816).

Seven of the inflammatory lesions classified as candidal leukoplakia lesions were noticed higher than the cutoff point and considered statistically false positive (non dysplastic lesions and have higher fluorescence than dysplasia) and three were lower than the cutoff point and considered true negative, while seven dysplasia lesions were considered true positive and three false negative.

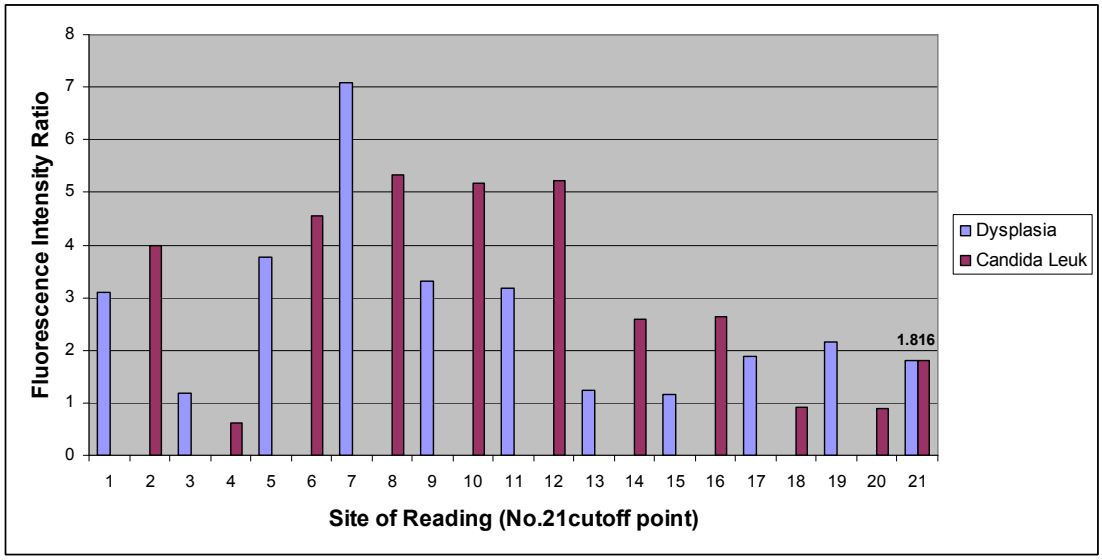


Figure 5.32. Comparison of the FIR measurements of the dysplasia and inflammatory lesions (candidal leukoplakia).

Using the value of one standard deviation (1.816) as a cutoff point to classify the FIR readings into true positive and negative results is shown in table 5.21. Seven of the inflammatory lesions representing candidal leukoplakia lesions were noticed

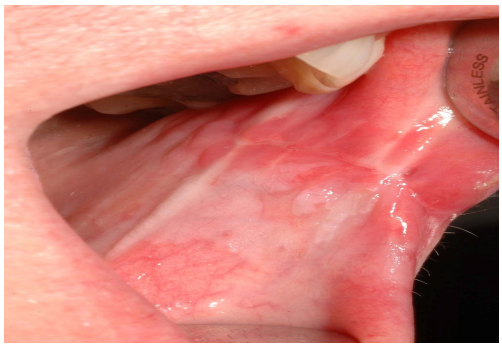
higher than the cutoff point (false positive) and three were true negative, while seven dysplasia lesions were considered true positive and three false negative.

It was noticed that the sensitivity yielded was 70% (true positive/true positive + false negative, 7/10) and specificity was 30% (true negative/true negative + false positive, 3/10). The PPV was 50% (true positive TP / (TP + FP) total positive, 7/14) and the NPV was 50% (true negative TN / (FN + TN) total negative, 3/6).

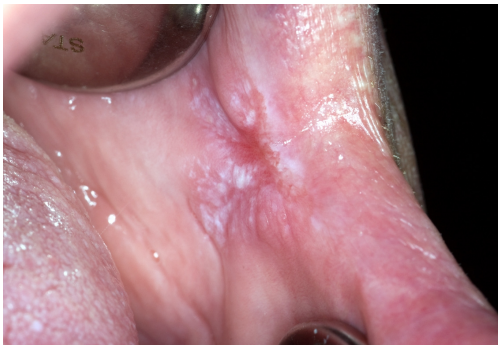
Site	Positive FIR	Negative FIR	Total
Dysplasia	7	3	10
Candidal Leukoplakia	7	3	10
Total	14	6	

Table 5.21. Sensitivity and specificity of FIR value in Discriminating between dysplasia and inflammatory (candidal leukoplakia) sites.

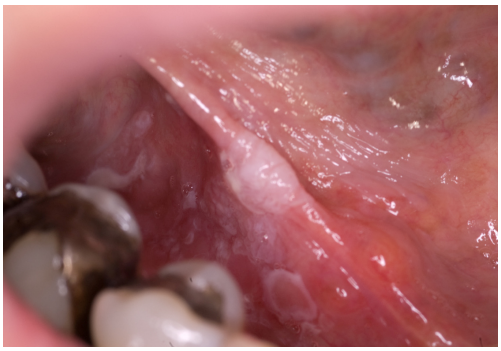
Figure 5.33. Clinically suspicious oral lesions (samples of detected lesions).



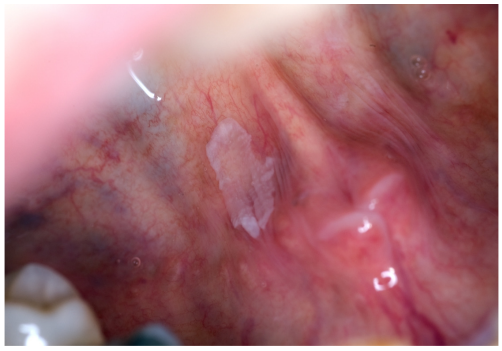
Dysplasia of the cheek (PDD006D)



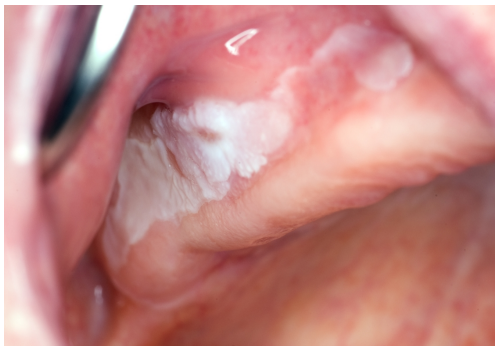
Candidal leukoplakia (PDD020D)



Squamous cell carcinoma (PDD022D)

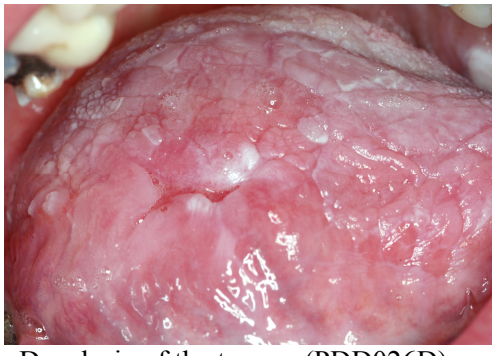


Lichenoid lesion (PDD021D)



Frictional keratosis (PDD008D)

Ulcerative keratotic lesion (PDD017D)



Dysplasia of the tongue (PDD026D)

Erosive (atrophic) lichen planus (PDD007)

Chapter 6

Discussion

The study protocol was designed to incorporate two investigation sites, Dundee and Glasgow. Compact fluorescence spectroscopy or OBS, as stated before, was developed by Glasgow University and used in the photobiology unit in Ninewells hospital for clinical research. The OBS used in this study has proved to be a reliable instrument that can be used to give an instant diagnostic reading from soft tissue body systems. Nadeau, *et al* (2002) has used the same instrument for in vivo point monitoring of aminolaevulinic acid (ALA)-induced protoporphyrin IX (PpIX) fluorescence and autofluorescence intensity measurements, as a non-invasive method of differentiating normal and cancerous tissue of the gastrointestinal tract. Their results illustrated the potential of the system to be used for fluorescence monitoring in a variety of clinical applications. Nadeau and his colleagues have also described the implementation of a compact fluorescence spectrometer in monitoring the photobleaching of ALA-induced PpIX in vivo on the skin of healthy volunteers (Nadeau V, *et al,* 2004). However, Ibbotson *et al,* (2006) found marked inter- and intra-subject variation in ALA-induced PpIX fluorescence in normal human skin. She related this to the body site and differences in the stratum corneum.

In their pilot study, Prof Ogden and his team used the instrument and 5-ALA as a non invasive tool in detecting oral cancer. They suggested that this instrument may be a valuable tool for detecting early oral cancer; however they recommended further studies to support their findings (O'Dwyer, *et al,* 2005).

After commencing this trial, the investigators observed variations in person-to-person fluorescence intensity peaks at each oral anatomical site in general. These variations were noticeable within a particular site in different persons. Furthermore

there were variations among different normal anatomical sites within the same person. As a result of this, an additional test (pilot study) was required to assess the reproducibility of the system in intra oral measurements at different oral locations.

Unlike most other organ systems suitable for fluorescence detection of malignancies, the oral cavity is lined with three mucosal types; masticatory, lining and specialised mucosa. The variations in the histological features of the oral mucosa might reflect differences in fluorescence spectral shape and intensity. For example, the cheeks, inner lip, soft palate, and floor of the mouth are lined with non-keratinized mucosa, while keratinized mucosa can be found on the hard palate, gingiva, and tongue. The presence of lingual papillae and taste buds makes the tongue coating mucosa unique in histological and anatomical features. Furthermore, the palatal mucosa and gingiva are supported by hard tissue or bone, which might increase the reflection of incident and fluorescent light. Therefore, it was considered worthwhile investigating the influence of oral anatomical location on fluorescence characteristics to see if baseline reading for a specific oral site could be obtained for comparison with a lesion under investigation. This study displays a map of the oral cavity fluorescence. To conduct such a trial in theory, the measurements should be compared with the histopathology, however it would be unethical and impractical to take biopsies from normal sites, therefore for this part of the study the measurements of normal anatomical sites depended on the clinical inspection of the investigators.

To our knowledge there were no previous studies assessing the measurements at oral sites using FIR measurements of the drug (enhanced) fluorescence. However

there was one study that measured the autofluorescence of the oral mucosa using the absolute fluorescence intensity (de Veld *et al,* 2003).

The OBS instrument was designed to measure the wavelength ranges from blue to red spectra i.e. for the measurement of the autofluorescence (green fluorescence) spectrum as well as the PpIX (red fluorescence spectrum). This could be obtained in two forms; a spectral line graph which appears on the screen and in real time as shown in figures 4.1 and 4.3 (Patients and Method section) and the ratio of any wavelengths required (by fixing the cursers on the X axis scale that exist with the line graphs) as shown in a slot on the screen of the computer. As mentioned in the review section several techniques have been proposed for the evaluation of fluorescence spectra. Principal component analysis (PCA) was the technique used in O'Dwyer's pilot study (O'Dwyer *et al,* 2008); however redundancy and transfer of the author limited the availability and the use of the software in the result analysis.

Fluorescence ratios are an approach used to avoid artefacts in the data. This technique has been extensively used for tissue characterization following the administration of fluorescent tumour markers (de Veld *et al* 2005; Sharwani *et al,* 2005). Red and green fluorescence peaks, and their ratio diagnostic algorithm were used to quantify the fluorescence intensities since the finding could be recorded and obtained from the instrument.

In statistics, normalization or transforms are usually applied so that the data appear to more closely meet the assumptions of a statistical analysis procedure that is

to be applied, or to improve the interpretability or appearance of graph figures (Bland, 1996a and b). In the result analyses in general, the comparison was done by including all the readings obtained without exclusion of the extreme values (outlier) or transforming the values. However, normalization (transforms) of the data and spectra linegraphs (in the result section figures 5.2, 5.4, 5.19, 5.22, and 5.25) were applied to give better result analysis interpretation.

The 1st pilot study showed the reproducibility of the instrument as a measurement device in intraoral application. In clinical practice variability might have occurred in some instances, because of some factors that impinge on the result outcome such as experience of the practitioner less so, more likely variation in the thickness of epithelium, under diagnosed systemic condition that influence fluorescence. .

The results were interpreted in two ways; the first was the traditional comparison between variables, which included statistical analysis of the measurement of the FIR at each site in relation with the other sites (and its significance). The second type of comparison was done spectrally, to show the average spectra line graphs readings at each site in relation to the average readings of the other sites.

These line graphs was representing the fluorophores after excitation at 405nm i.e. emission of light at different wavelengths. Several endogenous fluorophores emit light at the autofluorescence wavelengths; however PpIX was the only specific fluorophore whose emission was elucidated. Therefore, the shapes of the spectra line graphs that are displayed portray differences in cellular fluorescence emission, which in turn reflect differences in tissue structure.

6.1 Fluorescence Intensity Ratio (FIR) Measurement at the Oral anatomical Sites

It was thought initially that twenty four sites would be measured from each patient. This was taken from two patients (PDD004 and 5). However the procedure was impractical (time consuming and exhausting to the patients), hence the number of measurements taken were reduced.

To study the fluorescence of the oral cavity, ten sites were chosen to map out the fluorescence of the oral cavity. These sites were selected based on their location to include various oral mucosal types (keratinized, non keratinized and specialized) as fixed anatomical sites and easy to be accessed in the dark room in which the measurements would be taken. Thus the procedure of spectroscope probe application could be standardized.

The incisive papilla was a clear anatomical site at the anterior palatal mucosa and represents the most vascular in consistency and rarely involved by any oral lesions. Mid palatal mucosa right side and close to the mid palatine suture represented smooth keratinized mucosa and was easy accessed by the probe.

The tongue measurements were taken from the tip of the tongue 2mm and 3cm away from the border which represented the specialised mucosal coatings. The ventral tongue (5mm away from the border) was non keratinised mucosa and close to the floor of the mouth environment.

Floor of the mouth at mid Wharton's duct was fixed and an accessible site where bacteria and saliva are collected for a long period of time.

Two points were taken from the cheek representing the measurement of the fixed contralateral sites of the oral mucosa, in addition to detect internal consistency of the fluorescence measurement outcome.

The gingival site chosen was taken from the lower anterior region of the gingival mucosa at the top of the lower labial fraenal attachment. This site was easily accessed and representing attached gingival mucosa, regardless of whether the participant was dentate or edentulous.

The lower lip mucosal site was taken from the lower lip mucosa above the lower labial fraenal attachment. It was a lining mucosa which could easily be accessed by the probe.

Although our intention was to keep the fluorescence probe in the same position for all measurements, variations in positioning might have accounted for some of the variation in the fluorescence intensities. In addition the investigator was very conscious of not taking measurements from anatomical site involved by the lesion.

de Veld and her colleagues studied the autofluorescence intensity of the normal oral sites and discussed their results thoroughly. They anticipated that ratio-techniques and statistical methods applied to normalized spectra would be more accurate than techniques that rely on the absolute intensity for the diagnostics. These

techniques will reduce inter patient variability as well as irreproducibility, since Fluorescence Intensity Ratio seems more consistent than spectral shape or mere fluorescence intensity (de Veld *et al,* 2003). For this reason we have interpreted the result graphs in both normalised and none normalised forms (transformed data). Logarithm or natural transform method was applied to improve the data interpretation in box plot graphs as shown in figure 5.2.

Multicomparison classification for the ten anatomical sites has shown that fluorescence spectra obtained from the palate and the tongue were significantly different from those obtained from other anatomical locations at $p<0.001$ in particular when compared with the buccal mucosa. It is thought that the increase in cell layer thickness attenuated the tissue fluorescence emission and the green light in particular (Harries *et al* 1995; Stringer *et al,* 2008). This finding was in agreement with McGee (2008) who found that sites that are normally keratinized were distinct from nonkeratinized sites for a number of parameters and the data frequently clustered together. Schwarz et al, (2009) referred in their results to the variability between the keratinised and none keratinised mucosa and shallow verses deeper stroma in oral tissue sites.

Fluorescence of the palate and the tongue at the IP, MP, TT (2mm away from the tip) and DT (3cm away from the tip) sites were closely related statistically and spectrally. This may be due to the similar nature of the keratinised tissue coating or reflects on the microbial presence at these sites (Brazier, 1990; Konig *et al,* 2000). The FIR of the remaining locations (VT, FM, RC, LC, GN and LP) seems to be distributed similarly with no statistically significant difference.

In the result tables, the relation of the fluorescence intensity ratio (FIR) measurements and the normal oral sites was compared at two levels (0.05 and 0.001) of significance to explore in depth the comparisons between the ten oral sites measurements selected in this study.

The spectral distribution of DT and MP line graphs were lower in autofluorescence and drug fluorescence intensity peaks but the red/green ratio were similar which made the statistical analysis non significant. These findings suggest that the clinician should evaluate the peaks at two wavelengths (e.g. 500 and 635nm) and the ratio of the fluorescence peaks for each site in the comparison between the anatomical site and the normal and lesion site. This is in agreement with de Veld *et al,* (2003) who stated that total fluorescence intensities are highly inconsistent, both intra- and inter-individually and suggested the use of ratios of emission peaks at a single wavelength. Furthermore, in the same article, they anticipated that ratio-techniques and statistical methods applied to normalized spectra will be more accurate than techniques that rely on the absolute intensity for their diagnostic ability. It was thought this would reduce inter patient variability.

RC and LC were consistent in spectra line graph distribution i.e. there was no significant difference between left and right buccal mucosa. They had the highest autofluorescence and lowest drug (PpIX) fluorescence peaks which make them identical to the normal tissue (anticipated spectral linegraph shape in theory). There was variation in the fluorescence peaks in FOM as well as VT sites which could be due to the saliva and microbial flora accumulated that causes PpIX fluorescence emission.

The data analysis and the spectral line graphs of the anatomical sites could broadly be separated into two groups, the dorsal tongue & hard palate and the rest of the oral sites (mainly non keratinised). They could be used as a base line data for comparison with diseased tissue. A separate data base should therefore be considered for the palate and tongue. This finding was consistent with McGee and colleague who concluded that accurate spectroscopic detection of oral disease must account for spectral variations among anatomic sites in developing algorithm for the result analysis, and the nature regarding the keratinised and non keratinised mucosa (McGee *et al,* 2008, 2009).

Although the various oral sites showed certain similarities in FIR, that they vary with epithelial type (and hence were not identical) perhaps argues for a site specific table of normal values for comparison with lesions from the same oral site.

Based on these readings, it is suggested that the clinician should evaluate the lesions and compare them with the normal (contra-lateral) site in the same patient.

6.2 Fluorescence Intensity Ratio (FIR) Measurements in Relation to the Individual Characteristics

This study noted a statistically significant spectral difference between clinically normal oral mucosa at different anatomical sites. This suggests that compact fluorescence spectroscopy might be sensitive to individual characteristics since variation in FIR measurements between patients was noticed. No previous study has reported the effect of individual characteristics on the oral mucosa using drug

fluorescence; however de Veld and colleague reported their study on variation in autofluorescence intensity (de veld *et al*, 2004)

FIR measurements encompass both tissue autofluorescence and drug fluorescence peaks. Fluorescence spectroscopy is a non-invasive tool for the detection of alterations in the structural and chemical composition of cells ((Profio *et al,* 1984; Upile *et al*, 2007). Autofluorescence of tissues is produced by several endogenous fluorophores. These include fluorophores from tissue matrix molecules and intracellular molecules like collagen, elastin, keratin and NADH (Stringer *et al*, 2008). The presence of disease changes the concentration of these fluorophores as well as the light absorption and emission properties of the tissue. This takes place due to changes in blood concentration, epithelial thickness, nuclear size distribution, collagen and elastin content. It is therefore expected that the presence of disease will be reflected in fluorescence spectral shape and intensity.

Our hypothesis was that the non-pathological tissue alterations that cause these differences in FIR characteristics correspond to characteristics of the individual, such as age, gender, tobacco use, alcohol consumption, metabolic diseases and wearing of dental prosthesis.

Unfortunately the Glasgow team was not ble to collect all the patients information in spite of the limited number they recruited for the trial so the author included only the age and gender that were obtained from the case report forms (CRF) for the statistical analysis.

6.2.1 Comparison between Age Groups

Age groups were classified as over 50 years and under 50 years, since we have limited number of young participants age.

Previous studies on animal oral tissue samples taken from the palate, cheek and ventral surface of the tongue found no single age-associated change in epithelial structure or renewal common to all epithelia (Hill, 1988) In humans, it was concluded that the characteristic structural and functional changes that occur in skin and mucosa with age are not well defined (Hill, 2006).

Our results showed no significant difference in the FIR between the two age groups. However, in spectral analysis there was a slight elevation in the average drug fluorescence compared to autofluorescence in the young (less than 50 years) group. This could be attributed to the oral flora of young dentate patients participated in the trial compared to the older group many of whom were edentulous.

6.2.2 Comparison between Genders

The numbers of male and female participants were equal in distribution whilst the number of spectra is slightly different due to the missing values. In our results analysis for FIR, it was found that there was no statistically significant difference of the FIR between the gender groups. This finding shows there was no effect of the gender on the autofluorescence and drug fluorescence intensity ratio; however de Veld *et al*, (2004) reported that gender did have a significant influence on autofluorescence spectra. They explained that males' autofluorescence spectra

showed more blood absorption than females', while, the average autofluorescence spectra for men and women show equal peak intensities, indicating that the higher blood absorption is not a normalization artefact. Despite there being no substantial evidence for this in the literatures they explained this by differences in vascularisation between men and women or difference in epithelial thickness. We would consider the hormonal changes might have an impact on the oral mucosa of the females at certain conditions (due to physiological or medical management) (Friedlander, 2002) which later influence the autofluorescence peak; however such effect is limited on the FIR measurements.

Measuring the ratio of two wavelengths should be more reliable in detecting tissue changes (Sinaasappel and Sterenbor, 1993; Sterenborg, 1994). This measurement has been extensively used for tissue characterization following the administration of fluorescent tumour markers (Zheng *et al,* 2002, 2004).

Spectral linegraph shapes show the autofluorescence and drug fluorescence peaks of the male were higher than the female, however the ratios of the peaks seem similar in the values as shown in the figures (result section). The FIR measurement value distributions look consistent when comparing the inter-quartile range box plots of the two groups.

6.2.3 Comparison between Dental Prosthesis and None Prosthesis Wearers

A dental prosthesis is considered a factor that has influence on the oral flora. The microbiology of denture plaque and poor denture hygiene is associated with stomatitis (Candida infection). Photofrin demonstrated the susceptibility of C. albicans to its photodynamic effects (Chabrier-Roselló *et al,* 2005). These organisms may influence the mucosal tissue fluorescence. Our intention was to study the influence of the oral microbial flora of the participant wearing dental prosthesis on the FIR. We didn't find any significant difference between the FIR measurements dental prosthesis wearer and none prosthesis wearers.

The averaged spectral line graphs of the two groups showed a similar autofluorescence intensity level; however there was slight elevation in the non prosthesis wearer. This finding was unexpected since prostheses are known to cause alteration of the oral flora which would increase the red spectrum fluorescence.

6.2.4 Effect of Systemic Diseases

Metabolic diseases such as diabetes and hyperthyroidism can interfere with tissue activities which occur due to structural and/or biochemical alteration of the cells. The oral mucosa is commonly affected due to alteration of the tissue structure and function of saliva (Negrato and Tarzia, 2010; Venturi and Venturi, 2009). Diabetes is a metabolic disease associated with oral manifestations related to immune alteration, reduction in saliva flow rate and microbial infection. Cellular fluorescence of the diabetic skin has been studied. Ediger and his colleagues suggested that non-

invasive measurement of dermal fluorescence may be an effective tool to identify individuals at risk for diabetes and its complications (Ediger *et al,* 2009). We anticipated that oral mucosa fluorescence of diabetic patients would have an impact on the normal site readings which could influence the results analyses.

The number of participant recruited with systemic disease was low in comparison with none disease. Condition included diabetes and thyroid disease and the spectra obtained were utilised for the comparison.

The results showed there was no significant difference in the FIR measurements between the systemically diseased and none systemically diseased group. This means the autofluorescence and drug fluorescence (which reflect the cellular changes and presumably the bacterial flora) of the oral cavity were not affected by the systemic disease. This could be due to the fact that the patients were well controlled for their medical condition. Further studies are required involving those with uncontrolled diabetes (with different blood sugar levels) correlated with the FIR measurement to detect the effect of diabetes on mucosal fluorescence.

The spectral line graphs showed elevation of both autofluorescence and drug fluorescence in the systemic diseased group which compared with those lacking systemic disease. This difference could be due to variation in catabolic and anabolic cellular changes.

6.2.5 Effect of Smoking

The inflammatory effects of nicotine on the oral mucosa have been demonstrated (Dussor *et al,* 2003). The effect of cigarette smoking on the oral mucosa has been

assessed through the application of quantitative cytomorphometric analysis to smears obtained from clinically normal buccal mucosa. Ogden and co workers (1990) in their result analyses suggested that for normal buccal mucosa smoking does appear to influence cytomorphology. Smoking habit is considered as a commonality linking oral health with an increased risk for malignant disease (Seymour, 2010). However, oral epithelial dysplasia may exist in patients who do not smoke tobacco or drink alcohol (Jaber, 2010).

Our results for FIR showed no significant difference between smokers and none smokers FIR. However de Veld *et al*, (2004) found significant difference in the autofluorescence intensity peaks between smokers and non smokers while none significant difference between smokers and ex smokers. The controversy we have noticed in their results is the relatively no correlation between absolute smoking behaviour and fluorescence intensity and hardly any differences were observed between non-smokers and ex-smokers. They concluded that the effect of smoking on autofluorescence characteristics was of a temporary nature. In this study, we have included all smokers regardless of the number of cigarettes. There was no difference when compare smoker with non-smokers. The variation between our findings and theirs might be due to difference in study design and inclusion criteria. However, the spectral line graph of the smokers showed slight elevation in the red spectrum peak in comparison with none smokers. Hence the fluorescence intensity peaks indicated a slight effect of smoking on the oral tissue fluorescence (spectrally but not statistically).

Further studies should attempt to correlate the number of cigarettes consumed with the FIR measurements at a specific oral mucosal site since many people who develop oral malignant disease smoke and hence these influence need to be known and taken into account when assessing such conditions.

6.2.6 Effect of Alcohol Consumption

Alcohol has been recognized as an important risk factor for mouth cancer for almost 50 years. Ogden (2005) reviewed the effect of alcohol on cellular structure and function in reference to histologic and exfoliative cytologic studies of the oral epithelia. Alcohol may affect cell function of stem cells by both intracellular and intercellular pathways. In this thesis, alcohol consumption is based on the use or none use of alcohol regardless of the number of units drunk, because alcohol intake may vary and not be accurately reported.

Our results showed no significant difference in the FIR between the alcohol user and none alcohol user groups. In addition, the spectra line graphs showed closely related line distribution regarding the autofluorescence and drug fluorescence intensity peaks. This may be due the inclusion criteria that were applied in this study i.e. the amount of alcohol consumption were not graded to correlate the effect of the amount of alcohol with the FIR since this is considered as an oral malignant predisposing factor.

6.3 Fluorescence Intensity Ratio (FIR) Measurements in Detecting the Oral Lesions

6.3.1 Comparing the lesion and normal sites

The initial study protocol required multiple readings to be obtained from five sites (upper, anterior, lower, posterior and centre of the lesion) to cover the lesion size spectroscopically, however the punch biopsy of 5mm size would not include all the areas from which readings were obtained. In addition, in theory the biopsy reports would not interpret (necessarily) the cellular changes at the site the fluorescence reading is taken from. This is the limitation of this system (point monitoring system, unlike image monitoring system which could cover broader areas). Therefore, it would be more precise to take two readings (1^{st} and 2^{nd} readings) from 5mm size lesion (from the area which are clinically most suspected) to insure that the biopsied lesion area was measured "spectrally", for this reason the initial study design was modified.

1- Comparison between the 1^{st} readings, 2^{nd} readings and normal readings. The statistical analysis was done between the 1^{st}, 2^{nd} and the normal site readings. The Kruskal-Wallis procedure was chosen to compare between the three readings because of its reduced sensitivity to outliers and unbalanced data (=data with unequal sample sizes for the different groups). This reduced sensitivity occurs because the procedure performs an analysis of variance (ANOVA) on the ranks of the PC scores, rather than on their numeric values, like in standard ANOVA. A Multi comparison was performed on the Kruskal-Wallis results to test for any significant differences between different pairs of readings. It was found there was no statistically significant

difference between the 1st and 2nd readings which indicate the reliability of the readings (fluorescence intensity ratio measurements) in obtaining the readings from the lesion sites. In addition, there was a highly significant difference between the 1st reading from the lesion and normal site on one hand and 2nd reading from the lesion and normal sites on the other hand, which indicate the reliability of each reading in identifying difference between the lesion and the normal sites. It was presumed that using either set of lesion reading (FIR measurements) in the comparison would have similar result.

Spectrally, there was slight variation between the line graphs for autofluorescence level between the two lesion readings however they were overlapped on the PpIX peak which indicated little variation in the fluorophore and/or keratin fluorescence emission due to changes in the location measurement as shown in figure 4.18.

2- The other method of comparison was conducted between lesion sites and normal sites using the same sets of readings. This was done by merging the two lesion readings and compared with the normal site readings in order to assess the reliability of the OBS (spectroscopic measurements) in detecting any alterations in structure and composition of the tissue. The results showed that the FIR measurements were able to distinguish between the lesion and normal sites. This comparison was observed statistically and spectrally as well. This finding was in agreement with previous studies that used autofluorescence intensity (Gillenwater *et al,* 1998; de Veld *et al,* 2004) and photodynamic method in detecting the oral lesions (Swinson *et al,* 2006) and could conclude that the instrument was able to distinguish lesions in general from clinically normal tissue.

It has been noticed that porphyrin-like peaks "using autofluorescence spectroscopy" can appear in benign lesions (32%), dysplastic lesions (19%), malignant lesions (73%) and even occasionally in healthy oral mucosa (de Veld *et al,* 2003).

Selection of the cutoff point for the Statistical methods, in this study, was based on the concept which stated that the point may be used in addition to the clinical experience, analytical and empirical evidence for finding more reliable and valid cutoff point for classifying cases as positive or negative (Singh, 2007). Therefore the value of one SD of the merged groups' data was used to discriminate between the negative and positive readings.

The researchers often concentrate on the sensitivity and specificity of the test. However, the predictive values of the screening test are equally important as they measure the accuracy of the prediction made on a subject whose true condition is unknown i.e. the sensitivity and specificity was measured as a screening tool to test the percentage, while the predictive values test the proportions of the outcome (Severino, 2010), therefore, the two analyses were conducted in the result section.

The sensitivity of the fluorescence in distinguishing lesions from normal oral mucosa using compact fluorescence spectroscopy and 5-ALA was 59.5% with a specificity of 73.8%. This level of sensitivity and specificity was attributed to the method of including all types of oral lesions in the analysis, which involve benign hyperkeratosis that showed fluorescence emission behaviour (autofluorescence and PpIX peaks) similar to that of the normal tissue. Further studies classifying by type

of lesions to assess the sensitivity and specificity for each group should be undertaken.

6.3.2 Detecting Clinically Suspicious (Potentially Malignant) Oral Lesions

The method of collecting data was considered blindly, as neither the investigator who took the fluorescence measurements nor the pathologist who diagnosed the lesions were aware of each others outcome. The lesions classified as hyperkeratosis showed increased thickness of the keratin layer only, with no evidence of any inflammatory superimposition as described in the histopathological report. It was found that these lesions showed mild changes in the FIR measurements in real time spectrally, however the second group of lesion classified as inflammatory (includes lichen planus, lichenoid lesions and candidal leukoplakia) when the histology report mentioned inflammatory cell infiltrate and/or candidal infection without dysplastic changes. In clinical practice “spectrally” it was noticed that there was elevation in the FIR measurements, so that these lesions would have been classified differently from benign hyperkeratosis if blinded to the histopathological diagnosis. Dysplasia and neoplasia was classified into one group since staging of the disease was not included in the analysis and in addition the sample size was limited.

There was a statistically significant difference between the lesion and normal sites. This difference was explored spectrally "in real time "and normalized forms, as shown on the line graphs figures in the result section. The autofluorescence peak spectral line graphs were higher in normal sites and lower in benign hyperkeratosis, inflammatory lesion with dysplasia the least respectively. However the PpIX

fluorescence peaks were the lowest for the normal site and slightly increased in benign hyperkeratosis with highest peaks seen in inflammatory and dysplasia which overlapped one another. This was in agreement with the fluorescence spectral line graphs plotted to study the dysplastic changes of the tissue in the oral cavity (Gillenwater *et al,* 1998; de Veld *et al,* 2004) and in other organs (Stringer *et al,* 2008).

The specific alterations in tissue structure or biochemical composition causing the variation in fluorescence intensities of neoplastic and dysplastic tissue have not been fully elucidated. Endogenous fluorophores such as flavins, nicotinamide adenine dinucleotide (NADH), and collagen may undergo changes in quantity or form during neoplastic progression (Richards-Kortum and Sevick-Muraca, 1996). Gillenwater's group also found decreased peak intensities in pathologically abnormal vs normal tissues and increased intensity in the red region when comparing abnormal to normal tissues (Gillenwater *et al,* 1998).

A paper reviewing in vivo autofluorescence spectroscopy as a non invasive method in detecting oral cancer was published in 2005 (de Veld *et al*, 2005). They studied the autofluorescence and imaging for the early detection and classification of (pre) malignancies of the oral mucosa. They stated that sensitivity and specificity of the result analysis were varied in relation to the type of instrument used, method of detection, excitation wavelengths and classification algorithm. In discussing their results they thought that there was a problem in discriminating between benign lesions on the one hand and dysplastic and malignant lesions on the other hand. They have given explanation that tumours as well as benign and dysplastic lesions can be

of various degrees of keratinisation, hyperplasia and blood content and therefore, they will influence the shape and intensity of autofluorescence spectra (de Veld *et al*, 2005).

Our results showed that there was a significant difference in the FIR between dysplastic lesions and normal sites. The difference was clearly noticed in the data distribution as shown in box-plot and spectra linegraphs figures as well.

Detection of the classified lesions was further evaluated by analysing the sensitivity and specificity of the result. The 1st and 2nd lesion readings were utilized for this part of the study based on the fact that there was no significant difference between the two readings.

The sensitivity of the compact fluorescence spectroscopy and 5-ALA was 100% and specificity 100% in distinguishing dysplasia from normal sites due to specific cellular accumulation of PpIX.

The sensitivity and specificity values reduced when comparing between all inflammatory lesions (Lp, LL and candidal leukoplakia) with their normal sites. The low specificity values (55%) was noticed when the Lp&LL group was compared with the normal side which indicates that these lesions emitted light relatively similar to the normal tissues.

The results also showed there was statistically significant difference between dysplastic lesions and benign hyperkeratotic lesions with sensitivity was 77.7% and

specificity was 66.6%. This is due to relatively high cellular activity of the dysplastic or neoplastic lesions when compared with cellular activity of the benign lesions regarding the FIR measurements.

When assessing inflammatory lesions in comparison with dysplasia lesion, the statistical analysis including the various inflammatory lesions (lichen planus, lichenoid lesions and candidal leukoplakia) showed no statistically significant difference between this group and dysplasia, this could be explained by the heavy lymphocytic infiltration in the subepithelial connective tissue which can impact on tissue fluorescence (Krammer and Plaetzer, 2008) and the candidal hyphae superimposed on the lesion (as diagnosed by the histopathology reports). In isolating the classified inflammatory lesions (Lp&LL and candidiasis) the sensitivity and specificity varies in favour of dysplasia when compared with Lp&LL only.

As a matter of scientific curiosity for future studies, further group of lesion based on the presence of candida albican in the histopathology reports of the lesions has been classified and the sensitivity and specificity were calculated. The specificity of using fluorescence in detecting dysplasia from candidal leukoplakia was low (30%) in comparison with other inflammatory lesions (Lichen planus and Lichenoid lesions) (66.6%). Since fungal hyphae embedded in or on the surface of the oral lesions can induce synthesis of protoporphyrin IX from 5-aminolevulinic acid (Strakhovskaya *et al,* 1998), it could be anticipated that this might have an effect on the results.

The technique is highly sensitive to detect accumulation of PpIX inside the metabolically and structurally altered cells (dysplastic changes), however microbial presence in the oral cavity and to some extent the inflammatory infiltrates superimposing the lesion may have an affect on the statistical significance between the dysplasia and inflammatory groups. Therefore it could be concluded that Photodynamic Detection (PDD) method using compact fluorescence spectroscopy and 5-ALA was able to detect oral lesions from normal sites (but so is the naked eye!), however there was variation in the sensitivity and specificity in detecting a different pathological lesions. The technique was highly sensitive in detecting dysplasia from normal sites and the sensitivity varied in detecting classified lesions but unfortunately the technique is not able to discriminate reliably between dysplasia and inflammatory lesions whose clinical appearance can be very similar.

One point would be worth mentioning and supports the findings "practically " during conducting the trial. The author had written reports for a couple of lesions to the maxillofacial surgeon in Ninewells hospital describing the behaviour of some suspicious oral lesions “photodynamically” to assist in detecting the extent of the lesion and the highly suspicious areas for surgical removal. The reports were in details which encouraged the surgeon to refer more cases for investigation, but unfortunately the investigators had to comply with the MHRA recommendation to terminate the trial.

Chapter 7

Conclusion,

Limitation of the study

and Suggestions

7.1 CONCLUSION

This dissertation has investigated the use of the compact fluorescence spectroscopy together with the photosensitizer 5-aminolevulinic acid in assessing clinically suspicious oral lesions (photodynamic detection method) and the following conclusions can be drawn;

7.1.1 Pilot study

The pilot study confirmed the reliability and reproducibility of the use of the instrument for oral application with the photosensitizer 5-ALA.

No conclusion could be drawn as regards the reason for PpIX peaks from normal dorsal tongue. Some evidence for the influence of bacteria was obtained.

7.1.2 Main study

7.1.2.1 Fluorescence intensity ratio (FIR) measurement at the oral anatomical sites

The main aim of the study was to map the normal oral mucosa fluorescence intensity ratios (FIR) at ten different sites. The result showed that the oral sites could be classified into two main groups to be used as a baseline for comparison with lesionsat these sites; the dorsal tongue and palate as one group and ventral tongue, floor of mouth, cheeks, gingiva and lip as the other.

7.1.2.2 Fluorescence intensity ratio (FIR) measurements in relation to the participants individual characteristics

The aim of this aspect of the study was to determine the effect of individual characteristics which included age, gender, presence of dental prosthesis, systemic diseases, smoking habits and alcohol consumption on the (FIR) measurements. The results showed no effects of these factors on the readings obtained for the study, however there was slight variation spectrally but statistically the difference was not significant.

7.1.2.3 Fluorescence intensity ratio (FIR) measurements in detecting the oral lesions

In this study the aim was to determine whether the technique could detect potential malignant change in a range of oral mucosal lesions with particular reference to oral lichen planus.

7.1.2.3.1 Comparing the lesion and normal sites.

Comparison between 2 readings taken from the lesion and the normal corresponding site readings showed there was no difference between the 2 lesion readings i.e. intralesional consistency; however there was a difference when the lesion measurements were compared with the normal site readings

7.1.2.3.2 Detecting clinically suspicious (potentially malignant) oral lesions

The lesions were classified into three histological groups;

a- Hyperkeratosis showed increased thickness of the keratin layer only.

b- Inflammatory (includes lichen planus, lichenoid lesions and candidal leukoplakia).

c- Dysplasia (and neoplasia).

It was noticed that

- There was a difference in the FIR between the:

Dysplastic lesions and normal sites.

Dysplastic lesions and benign hyperkeratosis.

Inflammatory lesions and normal sites.

- However there was no difference between the:

Dysplastic and inflammatory lesions.

Inflammatory lesions and benign hyperkeratotic lesion.

Benign hyperkeratotic lesions and the normal sites.

7.1.2.3.3 Sensitivity and specificity of the use of Fluorescence Intensity Ratio (FIR) measurements in detecting oral premalignant (clinically suspicious) lesions

The sensitivity and specificity were calculated using the cutoff point (the value of one standard deviation of the compared groups) to discriminate the FIR measurements (Negative and Posative values) between the compared groups. It could be concluded that the technique was highly sensitive in detecting dysplasia from normal sites and the sensitivity is relatively varied in detecting different pathological lesions but unfortunately the technique is not able to discriminate reliably between dysplasia and inflammatory lesions whose clinical appearance can be very similar.

7.2 LIMITATION OF THE STUDY

The proposed recruitment (following statistical advice from Prof. Peter Donnan, University of Dundee) was a total of 50 patients, 25 at each of the two centres. Unfortunately, this target couldn't be achieved due to a number of factors; Numerous administrative and technical constraints that emerged after commencing the clinical work, such as management problem from the NHS who tried to impose limitations on when and where the compact fluorescence spectroscopy (OBS) measurements and biopsies could be taken, low referral of suitable patients due to competing pressure from other studies in the department, restricted availability of the OBS instrument due to its use in other trials, loss of staff having expertise in the use of the OBS system (Dr Martin O'Dwyer physicist at the University of Glasgow was made redundant and not replaced). Furthermore the preparations and consequences of the MHRA trial inspection meant that for some considerable time we couldn't recruit additional patients and the targeted number of patients could not be achieved due to the trial being prematurely closed on what was considered a technicality. The study had no QP release (Qualified Person) to confirm that the generic drug had not be tampered with during it's passage from it's site of production in a German factory to Ninewells hospital, Tayside.

Dundee (the centre where the author and the principal investigator resided) was able to achieve the target number and additional patients were recruited but only after having to wait to get permission from the REC, the MHRA and local R&D office following what is termed a substantial amendment to compensate for the shortfall from the Glasgow centre.

MHRA visit to Dundee

The University of Dundee, NHS Tayside, the Clinical Research Centre and Tayside Clinical Trials Unit (TCTU) officials were notified that they would be visited by the MHRA for inspection in May 2009. The PI, author and other investigators were informed that their trial had been chosen for particular attention and thus to prepare for the entire documentation to be checked by the inspectors. Ten trials were chosen for inspection with ours being one of six trials to be checked in detail. The MHRA have the authority to close down clinical trials. Tayside Clinical Trials Unit presented a series of seminars and workshops to train the investigators on how to conduct a CTIMP to the best standard and how to comply with the regulations of ICH GCP. The author had attended all these meetings, seminars and workshops.

The raw product of 5-ALA used in this trial was produced, signed and licensed in Germany. In subsequent meetings with the authorities in Dundee (prior to the MHRA visit) regarding the inspection, the trial pharmacist reassured the PI and the author of this thesis that although the drug was a raw material and prepared in Germany it should be acceptable.

On the day of the MHRA inspection of this study the papers were checked thoroughly and the inspectors asked for the QP signature in Dundee. Since there was no qualified person authorised to sign the drug release, the MHRA recommended terminating the trial unless QP release could be obtained.

Since Ninewells Hospital did not have a QP (and wouldn't fund this) the trial was withdrawn and no further patients could be recruited. This was due in part to the worry to contend this decision might lead to problem with Tayside going approved to be a recognized centre for such studies. A few days after the MHRA visit, a message was received informing Dundee that it had received provisional approval as a clinical trials centre, which subsequently received full approval. It established the Tayside Medical Science Centre (TASC) in 1st January 2010.

7.2 SUGGESTIONS FOR FURTHER STUDIES

During the study a number of issues arose, prompting consideration of the following studies, to improve our understanding of the system and its utility in diagnosing oral cancer at an early stage.

- Recruiting more malignant lesions as well as dysplastic lesions.
- Correlation of the stage of dysplasia with the FIR measurement, (mild, moderate and severe dysplasia, carcinoma in situ, early oral cancer and advanced oral cancer).
- Investigation of the effect of fungal (or bacterial) superimposition on FIR measurements.
- To recruit volunteers who do not have oral lesions in order to map the normal anatomical sites to improve the mapping of FIR measurements.
- To investigate why there is a high FIR measurement from the normal dorsal tongue mucosa.
- Expand the number of participants to include a wider age range, gender and race for comparison of the FIR measurements between groups.
- To study whether dental prostheses can effect the FIR measurements.
- To investigate whether systemic or metabolic diseases can interfere with the FIR measurements from normal oral mucosa.
- To investigate the correlation (if any) between the number of cigarettes consumed and FIR measurements from normal oral mucosa.

- To investigate the correlation (if any) between the amount of alcohol consumed and FIR measurements from normal oral mucosa.
- To investigate whether DNA ploidy analysis in conjunction with FIR measurements can improve the sensitivity and specificity of the technique particularly in respect of discriminating inflammatory from dysplastic lesions.

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Appendices